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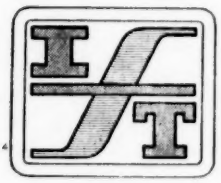

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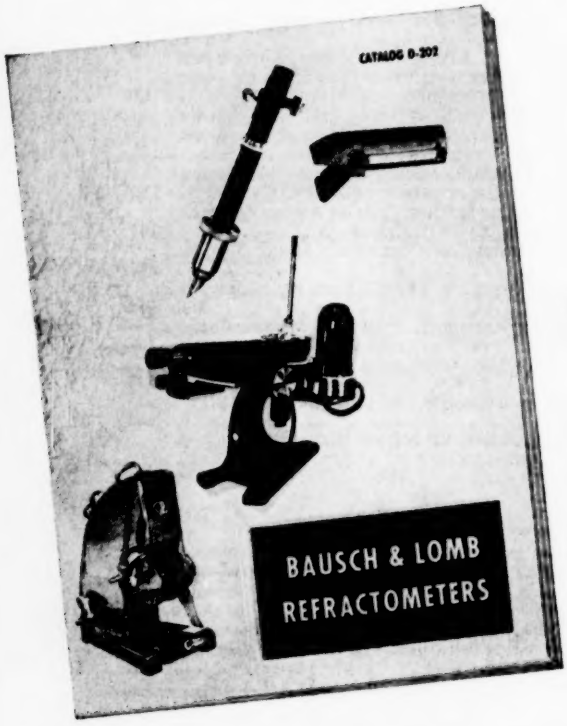

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THE PROBLEM OF HEAT INJURY TO DIETARY PROTEIN[a]

L. E. FRAZIER, P. R. CANNON, AND R. H. HUGHES
Department of Pathology, University of Chicago

(Manuscript received September 18, 1952)

The purpose of the following experiments was to secure additional information concerning the nature of heat injury to dietary protein. Samples of protein, amino acid mixtures or amino acid solutions, were subjected to heat treatment at different temperatures for differing periods of time. Mixtures of protein and carbohydrate or mixtures of amino acids and carbohydrates were compared with respect to their vulnerability to heat damage. In some instances the effects of amino acid supplementation, hydrolysis, pH, concentration of reactants, and differences between wet and dry heat were also studied.

EXPERIMENTAL

For the measurement of the extent of heat injury the rat-repletion method was used *(2, 13)*. This consists in the determination of the degree of protein repletion of adult, male, albino rats (Sprague-Dawley) which have been on a low protein diet until they have lost around 30 per cent of their initial weights. Such protein-depleted animals, when fed a daily repletion ration containing approximately 216 mg. of nitrogen, an adequate supply of vitamins and salts, and an energy potential of 48 cal., make average weight gains in 7 days of between 40 and 45 g. per rat. This represents the formation of new tissue protein (muscle, hemoglobin, plasma protein, etc.). If, however, the ration contains either a low-quality protein, or a high-quality protein which has been damaged by heat, or if it is deficient in one or more essential amino acids, the animals tend to eat poorly and to regain lost weight ineffectively. This method provides a rapid means of evaluating the nutritive quality of a ration in terms of its protein or amino acid potentialities. The composition of the rations is given in Tables 1 and 2.

The proteins under investigation were added to the basal ration at approximately a 9% level (N × 6.25). All rations were made isocaloric by substituting an equal amount of protein for dextrin. If the protein contained carbohydrate, as for example, when casein was used, allowance for this was made, as it was when mixtures of carbohydrate and protein were used. Two types of amino acid mixtures also served as substitutes for protein (Table 2). Mixture *A* was patterned after the amino acid composition of casein. The composition of amino acid mixture *B* differed from that of *A* in that tyrosine was transferred to the basal ration and the quantity of each essential amino acid was increased by 10%. At the same time the amount of nonessential amino acid nitrogen was decreased so that the percentage of nitrogen would remain the same as in mixture *A*. The amount of methionine was also increased to replace the small amount of cystine omitted.

For the determination of nitrogen balance, urines were collected on boric acid-impregnated filter paper. Feces were collected daily and placed in Kjeldahl flasks containing digestion mixture and concentrated sulfuric acid. Autoclaving for 4 hours promoted smooth digestion without foaming. Kjeldahl nitrogen determinations of the diets and diet-consumption data were used to calculate nitrogen intake. Fecal nitrogen of metabolic origin was not determined in these experiments; hence absorbed nitrogen was considered by us as the difference between nitrogen intake and total fecal nitrogen.

Samples subjected to dry heat were spread in a thin layer on a covered pan floating in a circulating, constant temperature oil bath. The temperature of the oil was recorded because of the difficulty in measuring the temperature of the sample itself. In general,

[a] This work was done in cooperation with the U. S. Navy Department, Office of Naval Research. We also acknowledge the cooperation of the Douglas Smith Foundation for Medical Research, University of Chicago.

TABLE 1

Percentage composition of rations used

	Depletion	Basal	Fibrin[e]	Casein[f]	Amino acid	Basal for use with amino acid-glucose solution
Carrots	30.0	.....	.....	.....	.....	.....
Corn oil	4.0	4.0	4.0	4.0	4.0	5.45
Cornstarch	42.0	.....	.....	.....	.....	.....
Dextrin	.....	70.0	61.0	60.44	61.0	67.27
Oleum percomorphum (drops)	0.3	0.3	0.3	0.3	0.3	0.4
Protein or amino acid mix	.....	.....	9.9	10.7	12.12	.....
Ruffex (Fisher)	5.0	5.0	5.0	5.0	5.0	6.82
Salt mix[b]	4.0	4.0	4.0	4.0	4.0	5.45
Tyrosine	.....	.....	.....	.....	.....	0.76
Vitamin mix (1)[c]	5.0	.....	.....	.....	.....	.....
Vitamin mix (2)[d]	.....	1.0	1.0	1.0	1.0	1.36
Water (cc.)	10.0	16.0	15.1	14.86	12.88	12.89
Grams fed per day	15.0	15.0	15.0	15.0	15.0	11.0

[b] Hawk and Oser *(6)* modification of Osborne and Mendel salt mixture with copper sulfate and zinc chloride added to make one per cent each of the trace elements.

[c] Wilson's liver concentrate 20.0, Standard Brands brewer's yeast No. 2019 40.0, sucrose 38.0, choline chloride 2.0, calcium pantothenate 0.004, pyridoxine HCl 0.004, riboflavin 0.01.

[d] Choline chloride 20.0, nicotinamide 0.3, calcium pantothenate 0.2, pyridoxine HCl 0.05, riboflavin 0.1, thiamin HCl 0.05, sucrose 79.3.

[e] Wilson Laboratories, Chicago.

[f] Nutritional Biochemicals Corp. (Vitamin Test).

TABLE 2

Mixtures of amino acids

	Mixture A, %	Mixture B, %	g. per 100 g. of ration of "Minimum Essential Amino Acids" (Steffee) *(12)*
DL Alanine	4.74	4.23	0.577
L Arginine HCl	4.20	3.75	0.511
DL Aspartic acid	5.35	4.77	0.649
L Cystine	0.30	.....	0.037
L Glutamic acid	20.08	17.96	2.443
Glycine	0.42	0.38	0.052
L Histidine HCl	2.86	3.28	0.193
DL Isoleucine	11.01	12.67	0.813
L Leucine	10.25	11.79	0.483
L Lysine HCl	8.02	9.22	0.530
DL Methionine	2.96	3.84	0.260
DL Phenylalanine	4.40	5.08	0.300
DL Threonine	6.61	7.60	0.573
DL Tryptophane	1.52	1.76	0.097
L Tyrosine	5.42	.....	0.659
DL Valine	11.86	13.66	0.680

the temperature of the sample tended to be from 5 to 10 degrees lower than that of the oil. Samples of fibrin, casein, amino acid mixture "*A*" and combinations of some of these with cornstarch, sucrose, glucose, or fructose were heated for 30 minutes or one hour at temperatures ranging between 230°F.(110°C.) and 410°F.(210°C.). Some were also autoclaved to determine the differences between wet and dry heat. Fibrin

heated to the point of injury was supplemented with essential amino acids (Table 2) in various combinations. Weight gains in 7 days of repletion, and nitrogen absorption values were used to evaluate the nutritive quality of hydrolysates or mixtures of amino acids.

Amino acid solutions were tested in 5% concentrations. These were autoclaved at 240.8°F.(116°C.) either with or without glucose, for varying periods. Similarly, such solutions were autoclaved at pH ranges of 7, 9, and 10. The effects of concentration of reactants were determined by making 100-g. mixtures of amino acids, glucose, and water. Equal weights of amino acids and glucose were mixed, then solutions were made in which 20, 30, 40, and 50 g. of the mixture were added to 80, 70, 60, and 50 ml. of water respectively. These were neutralized and autoclaved for 15 minutes at 240.8°F. (116°C.). After cooling, reneutralization, and dilution to volume, these were fed as drinking solutions to protein-depleted rats *(2)*.

Fibrin was chosen as the principal protein for study because of its relative purity and freedom from reducing substances. After heating for 30 minutes or one hour at temperatures ranging between 230° and 410°F.(110° and 210°C.), the fibrin was added to the basal ration at a concentration of 9.9%. This furnishes 216 mg. of nitrogen per rat day. These rations were nutritively effective in the repletion period in every instance in which the fibrin had been heated for 30 minutes at temperatures up to and including 374°F.(190°C.). However, heating for one hour at 374°F.(190°C.) caused definite deterioration; and at 392°F.(200°C.) and 410°F.(210°C.), nutritive impairment was more marked. In fact, heating for 30 minutes at 410°F.(210°C.) caused a complete loss of nutritive value of fibrin as protein (Table 3).

TABLE 3

The effect of dry heating of fibrin on its nutritive value as shown by the seven-day repletion weight changes, in grams. (Two rats per point.)

Temperature	266°F. (130°C.)	302°F. (150°C.)	338°F. (170°C.)	374°F. (190°C.)	392°F. (200°C.)	410°F. (210°C.)
30 minutes.....	42, 45	44, 49	40, 49	38, 39	30, 29	−6, 3
60 minutes.....	42, 47	37, 45	39, 41	23, 25	10, 12	−4, −9

With this evidence that dry heat at 410°F.(210°C.) for 30 minutes made fibrin essentially worthless as dietary protein, experiments were performed in an effort to ascertain whether the injury represented the actual destruction of essential amino acids or an inactivation whereby one or more essential amino acids became nutritively unavailable, as, for example, through the inability of intestinal enzymes to release them. Four samples of fibrin were prepared as follows: normal fibrin; fibrin heated at 410°F. (210°C.) for 30 minutes; normal fibrin, hydrolyzed; and fibrin heated at 410°F.(210°C.) for 30 minutes and then hydrolyzed. Hydrolysis was accomplished by refluxing a 5% solution of fibrin in 2 N sulfuric acid for 24 hours. These hydrolysates were then concentrated to 50% of the original volume in order to distill off, if possible, any objectionable side products. Barium hydroxide equivalent to the sulfuric acid used was added and the precipitate filtered and washed. After several washings decolorizing charcoal was used and removed and the mixture concentrated to a syrup at low temperature and pressure. The 4 samples were then added to the repletion rations in the amounts needed to furnish 216 mg. of nitrogen per rat day and fed to 4 groups of protein-depleted rats. To each ration containing hydrolyzed fibrin, tryptophane was added in the amount of 350 mg. per 100 g. of ration in order to replace that probably lost in the course of the acid hydrolysis. The results of the repletion tests (Table 4) demonstrate that the nutritive efficiency of the heated fibrin, although completely lost by the heat treatment, was restored by hydrolysis to approximately 65% of that of the unheated fibrin. It is evident, therefore, that a considerable proportion of the amino acids in the fibrin were not destroyed by heating but were inactivated or bonded in some way so that they could be released by acid hydrolysis but not by the action of intestinal enzymes. However, the fact that the nutritive potentiality was not completely restored by hydrolysis suggested the possibility that there may have been some destruction of amino acids as well.

TABLE 4

The effect of hydrolysis of fibrin heated (dry) at 410°F.(210°C.) for 30 minutes on 7-day nitrogen and weight balances. (Four rats per group.)

	N balance	Weight gain	N intake	N urine	N fecal	N absorbed
Unheated fibrin	1091	51	1628	327	210	1418
	1082	48	1601	299	220	1381
	1075	46	1594	286	233	1361
	1122	50	1575	242	211	1364
Unheated fibrin, hydrolyzed	977	48	1527	309	241	1286
	995	48	1483	278	210	1273
	878	47	1543	436	229	1314
	920	47	1504	333	251	1253
Fibrin heated at 410°F. (210°C.)	143	— 5	836	151	542	294
	71	— 5	866	127	668	198
	60	—10	730	129	541	189
	108	— 4	876	129	639	237
Fibrin heated at 410°F. (210°C.), and hydrolyzed	583	33	1447	564	300	1147
	404	30	1258	530	324	934
	653	36	1553	582	318	1235
	579	24	1369	524	266	1103

In order to learn whether one or more essential amino acids were involved in the combined injury and inactivation, samples of fibrin, heated at 410°F.(210°C.) for 30 minutes were supplemented with increasing numbers of minimum essential amino acids and all nonessential amino acids (Table 2) and added to the basal ration. The rations were nutritively inadequate except when all 9 essential amino acids had been added, indicating that some injury to and inactivation of all the essential amino acids in the fibrin may have occurred under the influence of heat.

In evaluating the consequences of heat injury to fibrin it was helpful to have a standard of reference, both in relation to weight gains and to nitrogen absorption. For this purpose 4 pairs of protein-depleted rats were fed repletion rations under the usual conditions of testing, but containing 4 levels of unheated fibrin viz., 3, 5, 7, and 9%. The results are seen in Table 5.

Having established the fact that dry heat at 374°F.(190°C.) for 30 minutes caused only slight nutritive deterioration in fibrin, an attempt was made to ascertain whether the heating of fibrin mixed with carbohydrate might more adversely affect its nutritive potentialities. In short, the object was to determine the effect of the Maillard reaction *(6)* on the nutritive quality of fibrin. As a preliminary test cornstarch was mixed with

TABLE 5

Seven-day repletion weight change on fibrin heated (dry) at 410°F.(210°C.) and supplemented with the following combinations of the minimum essential amino acids added to 100 g. of ration. (Two rats per group.)

Weight gain g.	Amino acids in supplement
0, —14	Hist.,
— 4, —10	Hist., Iso.,
—19, —23	Hist., Iso., Leuc.,
—16, — 9	Hist., Iso., Leuc., Lys.,
—20, —13	Hist., Iso., Leuc., Lys., Meth.,
—21, —16	Hist., Iso., Leuc., Lys., Meth., P.A.,
—17	Hist., Iso., Leuc., Lys., Meth., P.A., Threo.,
—21, —14	Hist., Iso., Leuc., Lys., Meth., P.A., Threo., Tryp.,
+12, +20	Hist., Iso., Leuc., Lys., Meth., P.A., Threo., Tryp., Val.

an equal amount of fibrin and heated at 374°F.(190°C.) for 30 minutes. The repletion test evidenced no greater deterioration than that observed with fibrin heated alone under similar conditions.

The heating of mixtures of fibrin and other carbohydrates, as, for example, glucose, fructose, or sucrose, caused differing effects. For example, a fibrin-sucrose mixture heated at 374°F.(190°C.) for 30 minutes became useless as a protein constituent of a repletion ration. Thus sucrose was injurious to fibrin under conditions wherein cornstarch was not. However, the heating of glucose-fibrin and fructose-fibrin mixtures brought about protein injury at lower temperatures than did heating with sucrose. In fact, the heating of mixtures of sucrose and fibrin for 30 minutes at 302°F.(150°C.) and 338°F.(170°C.) caused no protein damage, whereas a temperature of 302°F.(150°C.) caused definite damage to a mixture of glucose and fibrin, and a temperature of 266°F. (130°C.) caused damage to a mixture of fructose and fibrin (Tables 7, 8, 9). Inspection of the data in Table 9 in particular demonstrates the decreased intakes of nitrogen, the rises in fecal nitrogen, and the resulting decreases in absorbed nitrogen which were responsible for the poor weight responses of the depleted rats.

TABLE 6

The effect of variations in the amount of fibrin in the repletion ration in relation to 7-day weight gains and nitrogen absorption. (Two rats per group.)

Amount of fibrin in ration (N × 6.25)	3%	5%	7%	9%
Nitrogen intake, mg.	518 521	864 945	1257 1245	1610 1610
Fecal nitrogen, mg.	97 111	92 104	109 125	141 125
Absorbed nitrogen, mg.	421 410	872 841	1148 1120	1469 1485
Weight gain, g.	10 6	24 28	34 34	42 40

TABLE 7

Seven-day data on fibrin-sucrose mixtures heated for 30 minutes, dry heat. (Two rats per group.)

Temperature	302°F. (150°C.)	338°F. (170°C.)	374°F. (190°C.)	410°F. (210°C.)
Nitrogen intake, mg.	1524 1523	1511 1511	933 1210	934 911
Fecal nitrogen, mg.	120 120	138 136	359 431	367 390
Absorbed nitrogen, mg.	1404 1403	1373 1375	574 779	567 521
Weight change, g.	39 45	43 43	—8 —1	—8 —7

In other experiments casein was also subjected to dry heat. Four samples were tested; one unheated and 3 subjected to dry heat for 30 minutes at temperatures of 302°F.(150°C.), 338°F.(170°C.), and 374°F.(190°C.) respectively. After mixing into repletion rations at nitrogen concentrations of 1.44% (10.7 g. of casein) the 4 rations were fed to protein-depleted rats for 7 days. The results are shown in Table 10. Here it is seen that whereas the average weight gain with unheated casein was 41 g., the rats eating rations containing casein which had been heated at 338°F.(170°C.) gained only 28 g.; and at 374°F.(190°C.) there was weight loss. These inadequate repletion gains

TABLE 8

Seven-day data on fibrin-glucose mixtures heated for 30 minutes, dry heat. (Two rats per group.)

Temperature	293°F. (145°C.)	302°F. (150°C.)	311°F. (155°C.)	320°F. (160°C.)	329°F. (165°C.)	338°F. (170°C.)
Nitrogen intake, mg.	1455 1468	1415 1483	1318 1460	1444 1494	1324 1315	947 857
Fecal nitrogen	195 240	450 387	895 646	822 871	944 1032	773 665
Absorbed nitrogen	1260 1228	965 1096	423 814	622 623	380 283	174 192
Weight change, g.	36 37	25 21	3 31	17 23	5 —3	— 9 —16

TABLE 9

Seven-day data on fibrin-fructose mixtures heated for 30 minutes, dry heat. (Two rats per group.)

Temperature	266°F. (130°C.)	284°F. (140°C.)	302°F. (150°C.)	320°F. (160°C.)
Nitrogen intake, mg.	1525 1529	1561 1460	1184 1331	942 1120
Fecal nitrogen, mg.	210 175	305 275	331 483	318 422
Absorbed nitrogen, mg.	1315 1354	1256 1185	853 848	624 698
Weight change, g.	32 29	19 14	4 6	—7 —2

TABLE 10

The influence of dry heat at varying temperatures for 30 minutes on casein in relation to 7-day rat repletion and nitrogen absorption

Temperature	Unheated	302°F. (150°C.)	338°F. (170°C.)	374°F. (190°C.)
Nitrogen intake, mg.	1174 1500 1516	1362 1479	1449 1218	846 854 .....
Fecal nitrogen, mg.	207 233 201	240 245	348 295	586 547 .....
Absorbed nitrogen, mg.	967 1267 1315	1122 1234	1101 927	260 307 .....
Weight change, g.	41 41 42	36 38	33 22	—9 —7 .....

were associated also with a lessened dietary intake and a rise in fecal nitrogen. For example, in the animals eating the casein which had been heated at 374°F.(190°C.) the amount of absorbed nitrogen averaged approximately 280 mg. As can be seen from Table 11, this would correspond to ingestion of a ration containing between 3 and 5% of unheated casein.

TABLE 11

The effect of variations in the amount of casein in the repletion ration in relation to 7-day weight changes and nitrogen absorption values

Amount of casein (N × 6.25)	3%	5%	7%	9%
Nitrogen intake, mg.	307	523	1033	1468
	298	626	1204	1500
	.....	471	.....	1516
Fecal nitrogen, mg.	113	93	199	207
	109	151	221	233
	.....	91	.....	201
Absorbed nitrogen, mg.	194	430	834	1261
	189	475	983	1267
	.....	380	.....	1315
Weight change, g.	—1	4	30	41
	—5	10	32	41
	.....	4	.....	42

Although it is generally agreed that wet heat, especially in association with increased pressure, accelerates heat injury to protein, experiments were performed in which some samples of glucose-fibrin mixtures were dry heated at 235.4°F.(113°C.) in a jacketed autoclave for 5 hours and 16 hours, respectively, whereas others—in a watery paste—were autoclaved for 15 minutes, 30 minutes, 60 minutes, and 120 minutes at a pressure of 15 p.s.i.

When the mixtures which had been exposed to dry heat were tested by the usual repletion method, there was no evident nutritive deterioration whether measured by weight gains or by the amounts of fecal nitrogen (Table 12). In contrast, wet samples, autoclaved only 15 minutes evidenced definite nutritive deterioration and this became more pronounced with longer periods of autoclaving until, after 2 hours, the protein quality of the autoclaved samples was too low to promote weight gains at all during the repletion period.

When one compares the fate of nitrogen in animals fed the unheated fibrin-glucose mixture with that of the mixture autoclaved for 2 hours (Table 12) it is seen that whereas the nitrogen intake in the rats fed the former was about 1,750 mg. in contrast to about 1,200 mg. for the latter, the fecal nitrogen was only 246 mg. for the former and 643 for the latter. Thus the amount of absorbed nitrogen was around 1,500 mg. in the animals getting the unheated fibrin and only 558 mg. for those getting the autoclaved fibrin. Inspection of the data in Table 6 shows, moreover, that in this latter range of nitrogen absorption the animals might be expected to gain about 15 g. in 7 days. The even poorer repletion effects actually observed are probably due to limitations in caloric, vitamin, and mineral intakes as well as to partial or complete inactivation of one or more essential amino acids.

In order to determine whether essential amino acids were destroyed or inactivated, autoclaved samples of fibrin-glucose were again compared in 4 categories, viz., fibrin-glucose mixture, unheated; fibrin-glucose mixture unheated, hydrolyzed; fibrin-glucose mixture autoclaved for 2 hours at 235.4°F.(113°C.); and fibrin-glucose mixture autoclaved at 235.4°F.(113°C.) for 2 hours and then hydrolyzed. Results of the repletion tests and nitrogen absorption data are seen in Table 13. Although the animals receiving repletion rations containing hydrolysates of both the unheated fibrin and the autoclaved fibrin mixed with glucose did not undergo as effective repletion in terms of average weight gains as did those eating the unheated fibrin, nonetheless the performance in

TABLE 12

The effect of wet autoclaving vs. dry heating of fibrin-glucose for varying periods at 235.4°F.(113°C.) on the 7-day weight changes and nitrogen absorption values

	Weight change, g.	Absorbed nitrogen, mg.	Fecal nitrogen, mg.	Nitrogen intake, mg.	Number of rats
Unheated	46	1501	240	1741	2
	44	1506	252	1758	
	45	1503	246	1749	
Dry heat, 5 hours	44	1360	259	1619	3
	48	1474	245	1719	
	49	1448	224	1672	
	47	1427	243	1670	
Dry heat, 16 hours	46	1478	228	1706	3
	44	1437	257	1694	
	44	1445	261	1706	
	45	1453	249	1702	
Autoclaved 15 minutes	33	1251	308	1559	3
	28	1079	370	1449	
	29	1173	396	1569	
	30	1168	358	1526	
Autoclaved 30 minutes	5	888	376	1264	3
	5	1308	277	1585	
	13	1107	478	1585	
	8	1101	377	1478	
Autoclaved 60 minutes	7	829	461	1290	3
	8	943	642	1585	
	13	897	550	1447	
	9	890	551	1441	
Autoclaved 120 minutes	1	573	734	1307	3
	1	553	567	1120	
	—2	548	627	1175	
	0	558	643	1201	

each case was excellent. This indicates that autoclaving had not destroyed to an appreciable degree essential amino acids in combination with glucose, despite the fact that the autoclaved mixture evidenced marked nutritive injury as seen in the repletion test and by the fact that the rats ingesting this material lost about half of the nitrogen in the feces, accounting for their failure to gain weight effectively. It is again evident, therefore, that in the amino acid-sugar reaction accentuated by heating, essential amino acids were not destroyed but were inactivated.

Attention was next directed to the consequences of heat treatment applied to amino acid mixtures. Inasmuch as mixture *A*, when added to a basal ration, effects adequate protein repletion (36 g. weight gain in 7 days), heating of such a mixture should enable one to ascertain whether some essential amino acids are more vulnerable than others to heat.

A sample of mixture *A* was therefore subjected to dry heat for 30 minutes at 356°F. (180°C.) and added to the basal ration at a concentration of 1.44 g. of nitrogen per 100 g. of diet (12.12 g. of amino acids). The poor performance of the depleted rats in the repletion test (13 g. weight gain in 7 days) indicated heat damage to one or more essential amino acids. Since lysine is commonly assumed to be particularly vulnerable to heat it was decided to see whether its presence was necessary to the production of heat damage in general or whether a mixture devoid of lysine could also be damaged

TABLE 13

The effect of hydrolysis of fibrin-glucose mixture autoclaved for two hours at 235.4°F.(113°C.) on 7-day repletion weights and nitrogen absorption values

	Weight gain, g.	Absorbed nitrogen, mg.	Fecal nitrogen, mg.	Nitrogen intake, mg.
Unheated mixture	44	1414	199	1613
	47	1374	239	1613
	47	1355	233	1588
	53	1407	206	1613
	47	1401	210	1611
	48	1390	217	1607
Hydrolysate of unheated	42	1348	241	1589
	48	1352	230	1582
	43	1326	218	1544
	39	1366	198	1564
	39	1382	212	1594
	42	1355	220	1575
Autoclaved mixture	13	635	684	1319
	15	651	696	1347
	2	628	582	1210
	7	575	552	1127
	10	629	658	1287
	9	624	634	1258
Hydrolysate of autoclaved	44	1237	317	1554
	46	1281	282	1563
	44	1245	322	1567
	43	1200	379	1579
	28	1070	232	1302
	41	1207	306	1513

at the same temperature. The latter proved to be the case since rats gained only 15 g. in 7 days when fed the mixture heated in the absence of lysine and then supplemented with unheated lysine before feeding. It is of special interest, furthermore, that heating of lysine alone at this temperature and time did not affect its ability to supplement an unheated mixture of amino acids devoid of lysine and engender a satisfactory repletion (38 g. weight gain in 7 days).

Experiments were next performed in which amino acid solution *B*, containing a 5% concentration of the amino acid mixture, was autoclaved at 240.8°F.(116°C.) for as long as 2 hours in order to determine whether injury to amino acids in solution might occur. None occurred. This was determined by the following modification of the rat repletion method. The protein-depleted rats were given a daily offering of the amino acid solution containing approximately 216 mg. of nitrogen, supplied in drinking tubes. The non-protein basal ration was fed in dry form. Before starting the actual assay the animals were started on the unheated solution *B* and the basal ration for 3 days, and only the animals which accepted the solution were used for the assay. Under these conditions depleted rats will perform in essentially the same fashion as if all dietary ingredients were supplied in a single dry ration.

When glucose in 5% concentration was added to solution *B* and the mixture autoclaved for 60 minutes, no damage was evident as shown by rat repletion assay (Table 14). When, however, autoclaving was done for one hour at 240.8°F.(116°C.) at pH levels of 9 and 10, definite nutritive damage occurred when glucose was present, but not in its absence from the amino acid solution (Table 14).

Finally, a study was made of the possible role of concentration of reactants, using 100-g. mixtures of amino acids, glucose, and water. The mixtures were prepared so that 10, 15, 20, or 25 g. each of amino acids and glucose were stirred in 80, 70, 60, or

TABLE 14

Repletion weight gains on 5% amino acid solution B autoclaved at 240.8°F.(116°C.) for one hour at different pH's either plus (+) or minus (−) 5% glucose. (Three rats per group.)

pH	pH 7		pH 9		pH 10	
+ or − glucose	+	−	+	−	+	−
Three-day weight gain on unheated solution *B*	23	24	20	21	23	23
	18	21	22	21	26	22
	36	14	27	27	22	13
	26	20	23	23	24	19
Seven-day weight gain on test solutions	27	29	−10	36	−43	24
	36	30	− 9	36	−44	19
	10	31	− 9	33	−50	26
	24	30	− 9	35	−46	23

TABLE 15

Repletion weight gains on amino acid solution B autoclaved 15 minutes at pH 7 at different concentrations of amino acids, glucose and water. (Five rats per group.)

Weight in grams of amino acids,	10	15	20	25
glucose,	10	15	20	25
water.	80	70	60	50
Three-day weight gain on unheated solution *B*	26	22	21	22
	24	23	12	25
	28	25	22	16
	21	22	17	23
	19	25	23	20
	24	23	19	21
Seven-day weight changes on heated solutions	30	24	13	− 3
	37	19	23	−34
	32	18	7	−29
	30	14	9	− 1
	35	21	13	− 1
	33	19	13	−14

50 g. of water. After neutralization, these were autoclaved for 15 minutes at 240.8°F. (116°C.). They were then cooled, reneutralized, diluted to volume, and fed as drinking solutions to protein-depleted rats, together with the usual offering of the basal ration. As evidenced by repletion performances (Table 15), there was nutritive deterioration in all instances and this became progressively more marked as the concentration of reactants increased. At the highest concentration, in fact, all animals lost weight. It is evident, therefore, that the amino acid sugar combination is more likely to develop the higher concentration of sugar and amino acids subjected to moist heat, even if for only 15 minutes at pH of 7.0.

DISCUSSION

The protein-depleted rats, as used in these experiments, are well suited for the evaluation of protein quality in that they gain weight rapidly on foods containing adequate amounts of good quality protein but fail to gain if one or more essential amino acids are missing from the ration or have been inactivated during processing. Since industrial processing of foods is facilitated by employment of high temperatures and pressures it seemed desirable in our experiments to attempt to define some of the limits of

treatment within which food could be processed without injury to its protein components.

The general problem of heat injury to protein has been reviewed recently in an effort to summarize the principal factors concerned *(3)*. For example, it has been found that heating or hot alcohol extraction of liver caused a large decrease in biological value, but that when acid hydrolysis was employed, much of the biological value was restored *(11)*. Although Greaves, Morgan, and Loveen *(4)* were able to improve the protein quality of heated casein by the addition of lysine, Block, Breese-Jones, and Gersdorff *(1)* found the loss of quality was not due to the destruction of lysine. In fact, they were able to recover as much of this amino acid from heated as from unheated casein. These experiments as well as those of Mecham and Olcott *(7)*, in which it was found that proteolytic enzymes *in vitro* fail to digest heated protein, suggest that animals fail to thrive on heated protein largely because it has become resistant to the action of intestinal enzymes.

Pader, Melnick, and Oser *(10)* and Melnick and Oser *(8)* observed that the rate of pancreatic release of lysine from casein was decreased by heat processing so that the lysine was ineffective in the supplementation of the other amino acids of casein absorbed earlier in digestion. Microbiologically assayed lysine was not decreased but the rate of *in vitro* liberation of this amino acid was so decreased that lysine supplementation was required for the utilization of the casein. In fact, the possibility that the lysine was liberated too late to be absorbed was thought likely.

Our experiments (Table 13) point in the same direction. Thus autoclaving a fibrin-glucose mixture at 235.4°F.(113°C.) for 2 hours increased the waste fecal nitrogen and decreased the amount of weight recovered in 7 days whereas hydrolysis restored almost completely the potentiality of the amino acids for permitting weight gain. In contrast (see Table 4) dry heating of fibrin at 410°F.(210°C.) for 30 minutes was more severe than autoclaving in that the animals not only lost weight during repletion but hydrolysis did not restore the utilizability of the protein to its former level. This indicates the possibility that essential amino acids have been destroyed, giving rise to an incomplete amino acid mixture which could not be utilized and was therefore excreted. Under the conditions of these experiments, the impaired utilization of amino acids is attributable to indigestibility, or variation in rate of digestion which resulted from temperatures of heating lower than those required for the destruction of amino acids.

The Maillard reaction *(6)* is a potent cause of nutritional heat damage to protein. Sucrose, however, is not a reducing sugar yet enters this reaction, presumably because it decomposes at 366.8°F.(186°C.). The resulting reactive fragments combine rapidly with fibrin when it is heated to 374°F. (190°C.) to render it nutritionally ineffective.

The cyclic forms of glucose and fructose presumably achieve their reducing potentiality by being in equilibrium with an acyclic aldose isomer. As the temperature rises the rate of reaction between the reducing aldose and the reactive groups of proteins increases. At higher temperatures the hexose splits into 2 triose fragments thus increasing the concentration of

available reducing compounds and hastening the binding of amino acids. Cornstarch, on the other hand, does not decompose at 410°F.(210°C.) and therefore does not furnish reducing compounds which may participate in the Maillard reaction.

Overby and Frost *(9)*, autoclaved a solution containing 5% glucose and 5% protein hydrolysate and withdrew samples at intervals for analysis. After autoclaving for 60, 80, or 100 minutes they found significant decreases in rat repletion responses in contrast to our finding that solution B was not injured by autoclaving for 2 hours in a 5% glucose solution. The loss of nutritive quality in the former experiment they believed was largely due to the low level of tryptophane in the original solution. This level dropped to nearly half the original value after 80 minutes' autoclaving.

Table 14 shows, however, that an elevation of pH speeded the nutritive degradation of an autoclaved amino-acid-glucose mixture probably because at elevated temperatures glucose in alkaline solution undergoes fission to produce reducing triose derivatives which can react with greater quantities of free amino groups.

Several of the techniques which can be used to increase the speed of chemical reactions are illustrated in the experiments reported here: (a) elevation of temperature, (b) stirring of reagents, (c) increase in concentration of reagents and (d) introduction of a catalyst. These experiments indicate that nutritional heat damage to protein can occur with dry heat in the absence of carbohydrate but that autoclaving in the presence of reducing sugars in an alkaline medium or in high concentration of reagents can cause protein deterioration at considerably lower temperatures.

SUMMARY AND CONCLUSIONS

Experiments were performed in an effort to compare the effects of dry heat and of autoclaving upon nutritional damage to protein in the presence or absence of certain carbohydrates, with altered pH's, or with varied concentrations of reactants and to learn the effects of hydrolysis on amino acid supplementation in restoration of lost quality. The rat repletion method was used. Nitrogen intake, fecal nitrogen values, and degrees of protein repletion indicated that the chief cause of nutritive failure was indigestibility of heated protein.

Failure of fibrin to engender protein repletion was seen when it was treated as follows:

(a) Heated for 30 minutes at 410°F.(210°C.) with dry heat.

(b) Mixed with equal parts of the following carbohydrates and heated with dry heat for 30 minutes at the following temperatures:
  (1) cornstarch, 410°F.(210°C.),
  (2) sucrose, 374°F.(190°C.),
  (3) glucose, 329°F.(165°C.),
  (4) fructose, 320°F.(160°C.).

(c) Mixed with equal parts of glucose and autoclaved wet for 120 minutes at 235.4°F.(113°C.).

The loss in protein quality produced in fibrin dry-heated at 410°F. (210°C.) was not restored by supplementation with various combinations of the minimum quantities of the essential amino acids until all 9 were present (Table 2).

Hydrolysis, moreover, restored a large fraction of the protein quality lost from dry-heat inactivated fibrin or from a fibrin-glucose mixture inactivated by autoclaving.

Both casein and casein mixed with cornstarch were inactivated by dry-heating at 392°F.(200°C.) for 30 minutes. This temperature is lower than the temperature of inactivation of fibrin presumably because of the presence of the reducing sugar, galactose, in the casein.

A mixture of amino acids heated with dry heat at 356°F.(180°C.) for 30 minutes became nutritionally ineffective for protein repletion. This inactivation temperature is lower than that of fibrin because of the presence of a large number of free reactive groups on the amino acids.

Autoclaving a 5% solution of amino acids in the presence of an equal concentration of glucose at 240.8°F.(116°C.) caused no nutritional damage although the heating was continued for 120 minutes. If, however, the autoclaving was done at pH 9 the mixture was completely inactivated after only 60 minutes' heating. As the concentration of reactants was increased the length of time of autoclaving the mixture of amino acids and glucose to produce nutritional damage decreased.

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LINOLEATE OXIDATION CATALYSTS OCCURRING IN ANIMAL TISSUES

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Much effort has been devoted to the identification of the catalysts in animal tissues which are responsible for the oxidation of unsaturated fats because of their pertinence to food preservation and nutrition. Since the only known enzyme which catalyzes the direct reaction of unsaturated fats with oxygen is lipoxidase, some investigators have suggested that this enzyme may be one of the unsaturated fat oxidation catalysts present in animal tissues *(9, 10, 11)*. The enzyme lipoxidase is known to occur widely in plant tissues *(8)* but its presence or absence in animal tissues has remained a perplexing problem. On the other hand, the heme compounds, myoglobin, hemoglobin, and the cytochromes occur in fairly large amounts and their ability to catalytically oxidize the unsaturated fats is well known *(3, 7, 13)*.

The differentiation of catalysis of unsaturated fat oxidation due to heme compounds from the catalysis due to lipoxidase has been one of the major difficulties. In studies of fat oxidation in hog tissue Lea *(10)* first suggested that an enzyme like lipoxidase might be involved. Watts and Peng *(16)* found the catalysis of fat oxidation by hog tissue extracts could be explained by their content of hemoglobin and myoglobin. Based on the observation that bacon extracts freed of hemoglobin still retained a heat labile catalyst that caused the oxidation of unsaturated fats, Reiser *(11)* concluded that an enzyme system may be responsible. In studies relative to the destruction of carotene in the presence of methyl linoleate, Hove *(9)* found that rat liver and stomach mucosa contained an unsaturated fat oxidase system which did not appear to involve catalysis by heme compounds since muscle was not an active catalyst. Hove compared the fat oxidation catalyst of rat stomach with soybean lipoxidase but did not arrive at a conclusive result. A fatty acid oxidase system was demonstrated in poultry fat by Ellman and McLaren *(6)*; however, they did not determine the nature of the catalysts involved. Banks *(1, 2)* found a heat labile catalyst in herring muscle which promoted oxidation of herring oil. Later *(4)* he demonstrated that the catalyst was a heme protein. Brocklesby and Rogers *(5)* studied the destruction of vitamin A by extracts of salmon liver and indicated that an unsaturated fat oxidase system may be involved.

A method allowing the separation of lipoxidase and heme protein catalysis has been used recently to study linoleate oxidation catalyzed by hog tissue extracts *(15)*. No enzyme system like the well characterized lipoxidase could be demonstrated in hog muscle or adipose. In this previous study it was found that heme compounds catalyzed the oxidation of linoleate only in colloidal or heterogeneous systems; however, lipoxidase catalyzed the oxidation of both colloidal linoleate and homogeneous lino-

leate. By the determination of the initial rate of oxygen absorption of colloidal linoleate substrate catalyzed by tissue extracts the potency of the combination of both types of catalysts can be found. Any oxygen absorption by homogeneous linoleate catalyzed by tissue extracts indicates the possible presence of lipoxidase.

In the present investigation the catalysis of linoleate oxidation by extracts of various animal tissues was studied to determine the presence or absence of the enzyme lipoxidase and to compare the rate of catalyzed linoleate oxidation with the equivalent hemin content of the tissue extracts.

EXPERIMENTAL

Materials. Tissue extracts were prepared from pooled samples of the larger animals or from the whole tissue of small animals so that a representative sample was obtained for analysis. The tissue was disintegrated in a blendor with a double weight of distilled water until a homogeneous slurry was produced. This mixture was resolved by centrifugation at 850 × gravity, and the aqueous extract containing the active catalysts was removed. The aqueous extracts were stored in small vials at −18°C. until ready for use. Dilutions, as necessary, were made with distilled water immediately before the measurement of linoleate oxidation and hemin content.

A lipoxidase preparation was obtained by aqueous extraction of defatted soybean meal. The potency of the lipoxidase preparation was 30 units per ml. according to the assay of Bergström and Holman *(4)*.

The colloidal linoleate consisted of 7.1×10^{-3} *M* sodium linoleate suspended in 0.1 *M* phosphate buffer at pH 7.0. Homogeneous linoleate consisted of 9×10^{-4} *M* sodium linoleate dissolved in 0.1 *M*. ammonium hydroxide–ammonium chloride buffer at pH 9.0. The linoleate substrates were protected from oxidation during preparation and frozen storage by an atmosphere of nitrogen.

Procedures. It was previously ascertained *(15)* that oxygen absorption measurement by conventional Warburg technique could be used to give a valid determination of the rate of linoleate oxidation. For oxygen absorption measurements 3 ml. of buffered linoleate substrate were placed in the main compartment and 1 ml. of catalyst was placed in the side arm to be mixed after equilibrium to a temperature of 10°C.

The equivalent hemin concentration of the heme compounds occurring in the animal tissue extracts was determined by reacting 5 ml. of diluted tissue extract with 0.5 ml. 50% aqueous potassium hydroxide and 0.25 ml. pyridine and measuring the alkaline pyridine hemochromogen thus formed by its absorption at 560 mμ. Crystalline hemin was employed as a standard.

RESULTS

The results of this investigation are tabulated in Table 1. Crystalline hemin catalyzed the oxidation of colloidal linoleate at a rate directly proportional to the square root of the hemin concentration *(15)*. Hemin did not catalyze the oxidation of homogeneous linoleate at an appreciable rate. The small and variable rate of 0 to 2 cubic millimeters of oxygen per hour in homogeneous linoleate was probably due to the presence of some colloidal linoleate in the homogeneous preparation, but can be considered insignificant when compared to the initial reaction rate in colloidal linoleate.

In contrast, lipoxidase is active in both colloidal and homogeneous linoleate, but the greater solubility of linoleate at pH 9.0 increases the rate of catalysis *(14)*. Data in Table 1 indicate that the amount of lipoxidase necessary to give a large rate of oxidation of homogeneous linoleate is very small. The soybeans used as a source of this lipoxidase contained 300 units per g., and other leguminous plants would probably contain not less than 10 units per g. *(12)*. With this present method of assay and concentrations of tissue of 0.13 g. per ml. a large initial oxidation rate should result if animal tissues contain 0.2 unit lipoxidase per g.

In the presence of heme compounds of some of the animal tissues assayed, a small concentration of lipoxidase may give a much larger initial rate of linoleate oxidation. Data are presented for the cases of rat stomach extract and rat muscle extract with added lipoxidase. This increased rate of oxygen absorption may be due to heme compounds caus-

TABLE 1

Rate of linoleate oxidation catalyzed by hemin, lipoxidase, and animal tissue extracts

Catalyst	Concentration of catalyst in each Warburg flask	Equivalent hemin concentration in each Warburg flask Molar $\times 10^{-6}$	Initial reaction rate Cu. mm. O_2 per hour	
			Homogeneous linoleate 9×10^{-4} *M* sodium linoleate pH 9	Colloidal linoleate 7×10^{-3} *M* sodium linoleate pH 7
Crystalline hemin	0.002 mg.	0.77	0 to 2	20
Crystalline hemin	0.004 mg.	1.54	0 to 2	28
Crystalline hemin	0.010 mg.	3.83	0 to 2	38
Crystalline hemin	0.020 mg.	7.66	0 to 2	54
Crystalline hemin	0.030 mg.	11.50	0 to 2	58
Crystalline hemin	0.050 mg.	19.18	0 to 2	76
Crystalline hemin	0.075 mg.	28.76	0 to 2	78
Crystalline hemin	0.100 mg.	38.30	0 to 2	86
Lipoxidase	0.025 unit per ml.	0	102	....
Lipoxidase + rat stomach	0.025 unit per ml. + 0.13 g. per ml.	1.2	96	144
Lipoxidase + rat muscle	0.025 unit per ml. +0.013 g. per ml.	1.2	146	140
Rat stomach	0.13 g. per ml.	1.2	0.5	28
Rat liver	0.013 g. per ml.	1.5	0.3	92
Rat liver	0.007 g. per ml.	0.8	0	60
Rat kidney	0.013 g. per ml.	20.3	1.2	152
Rat heart	0.013 g. per ml.	15.3	0.6	100
Rat muscle	0.013 g. per ml.	1.2	0.6	36
Cattle muscle	0.13 g. per ml.	43.0	0.5	110
Chicken muscle	0.13 g. per ml.	3.1	1.4	35
Chicken fat No. 1	0.13 g. per ml.	0	0.7	0
Chicken fat No. 2	0.13 g. per ml.	0	0	3.0
Turkey muscle	0.13 g. per ml.	19.2	1.7	72
Turkey fat	0.13 g. per ml.	0 (?)	0.3	16
Catfish muscle	0.13 g. per ml.	19.6	2.0	100
Halibut muscle	0.13 g. per ml.	3.1	1.7	5.3
Mackerel muscle	0.13 g. per ml.	12.7	2.7	115
Salmon muscle	0.13 g. per ml.	5.3	0.5	26
Shark muscle	0.13 g. per ml.	11.5	1.7	225
Smelt muscle	0.13 g. per ml.	9.6	0.8	7
Tuna muscle	0.06 g. per ml.	14.6	2.0	127

ing a breakdown of the linoleate peroxides and thereby accelerating linoleate oxidation. Heme compounds could also be co-oxidized in the lipoxidase-linoleate system. Animal tissue extracts plus added lipoxidase have consistently caused a large initial oxidation of homogeneous linoleate indicating that the animal tissues do not contain inhibitors for lipoxidase which could obscure this test for its presence.

From these results, it appears that if lipoxidase occurred in any significant amount in animal tissues its presence could be detected by using linoleate as a substrate. It is important to emphasize that this is only a method of detecting the possible presence of lipoxidase, and other studies would be necessary to characterize a catalyst which could cause oxidation of homogeneous linoleate.

For all the animal tissues assayed the initial rate of oxidation of homogeneous linoleate was very small, being in the range given by crystalline hemin. It was therefore concluded that lipoxidase was not present in significant amounts in any of these tissues.

The fact that the heme compounds in the animal tissue extracts can give a rapid catalytic oxidation of colloidal linoleate is amply demonstrated. On the basis of the equivalent hemin content of animal tissue extracts the catalysis of colloidal linoleate oxidation is of the same order of magnitude as that given by crystalline hemin, however

no good correlation exists between the hemin content and the rate of linoleate oxidation. On the average the tissue extracts gave a higher catalysis than would be expected from their equivalent hemin content alone.

DISCUSSION

The results of this investigation indicate that the predominant catalysts for unsaturated fat oxidation which occur in animal tissues are the heme compounds. This is in agreement with the conclusions of Watts and Peng *(16)* and Holman and Bergström *(8)*. Although good evidence was found for the absence of the enzyme lipoxidase, the possibility of other enzymic fat oxidation catalysts has not been excluded. On the contrary, the fact that tissue extracts gave a higher average catalysis than equivalent amounts of crystalline hemin shows that other catalysts besides heme compounds are probably present.

Since animal tissue extracts contain many catalysts and inhibitors, it was not surprising that the catalyzed linoleate oxidation cannot be well correlated with the hemin analysis. The elucidation of the mechanisms of unsaturated fat oxidation in animal tissues depends upon further research of the individual tissue catalysts and antioxidants.

SUMMARY

A method for the determination of lipoxidase in the presence of heme compounds has been described. Application of this assay has indicated the absence of lipoxidase in the tissues of the rat, cattle, chicken, turkey, and various fishes.

Most of the linoleate oxidation catalyzed by animal tissue extracts could be ascribed to their content of heme compounds.

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THIAMINE, RIBOFLAVIN, AND NIACIN CONTENT OF ORGAN MEATS[a]

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Some data are available on the thiamine, riboflavin, and niacin content of muscle cuts and certain organ meats from swine, cattle, and sheep *(1-12)*, but a comprehensive study of the vitamin content of organ meats is not readily available. The purpose of this study was to determine the thiamine, riboflavin, and niacin content of representative, composite samples of beef, pork, and lamb organ meats that were obtained directly from two meat-packing companies within 48 hours after the animals were slaughtered.

EXPERIMENTAL METHODS

Samples for the experiment were obtained from 2 meat-packing companies and were representative of the October-November slaughter period. The samples were taken from young lambs, hogs averaging 225 pounds in weight, and beef graded good (source 1) and commercial (source 2). Additional samples of liver were obtained from old sows (325 pounds in weight) and from old cows (cutters and canners). Whole organs, or in the case of liver 1- to 2-pound portions, were obtained from 10 different animals, composited and finely ground through an electric grinder. Extraneous fat and connective tissue that accompanied the sample were removed before grinding. Each sample was well mixed and a portion taken for vitamin analyses. Proximate analyses were made on a duplicate sample. The remainder of the sample was lyophilized, ether extracted, ground, and stored in the refrigerator for future research.

The fresh samples were analyzed for proximate composition and for thiamine, riboflavin, and niacin. Thiamine was determined fluorometrically, riboflavin microbiologically and fluorometrically, and niacin microbiologically. These methods have been discussed earlier *(10)*.

RESULTS AND DISCUSSION

The sampling technique provided a good, representative sample within the limits of one slaughter season and one section of the United States. The organs studied included brain, spleen, pancreas, liver, heart, lung, and kidney. Liver from old cows and sows was also selected in order to evaluate the influence of age of the animal on nutrient composition of the liver. The proximate composition of the samples is given in Table 1.

The thiamine, riboflavin, and niacin content of the samples is given in Table 2. The values for beef liver and heart compare favorably with those found in the literature *(1, 3, 4, 6)*. Pork muscle cuts are high in thiamine *(11)* compared to beef or lamb cuts. The thiamine content of pork organ meats was found to be essentially the same as that for beef and lamb organ meats. In general, heart, kidney, and liver contain approximately twice as much thiamine as brain, spleen, lung, and pancreas. The riboflavin and niacin content of liver is much higher than that of other organs. The values for riboflavin range from 0.2 mg. per 100 g. for brain to 3.9 mg. per 100 g. for lamb liver. The niacin content of the organs studied varied from approximately 3.0 mg. per 100 g. for beef pancreas to approximately 18 mg. per 100 g. for pork liver.

It is of importance to point out that on an overall basis the data for the thiamine and riboflavin content of the composite samples obtained from 2 sources were in excel-

[a]Journal paper No. 57, American Meat Institute Foundation.

TABLE 1

Proximate analyses of organ meats

Organ	Source	Protein [b]	Moisture	Ether extract	Ash
		% in sample			
Beef liver	1	19.0	69.2	5.6	1.1
Beef liver	2	20.0	71.6	1.3	1.3
Lamb liver	2	20.6	71.5	0.4	1.4
Pork liver	1	20.5	72.0	4.8	1.4
Pork liver	2	20.5	73.4	2.3	1.5
Beef liver (cow)	1	20.9	71.7	1.6	1.4
Beef liver (cow)	2	19.5	73.4	0.2	1.3
Pork liver (sow)	1	21.8	72.0	2.8	1.4
Pork liver (sow)	2	18.9	72.8	1.8	1.9
Beef kidney	1	16.9	78.4	3.1	1.0
Beef kidney	2	15.7	73.1	9.0	1.0
Lamb kidney	2	15.6	80.2	2.9	1.0
Pork kidney	1	15.1	79.6	3.9	1.0
Pork kidney	2	16.0	76.1	6.8	1.1
Beef heart	1	15.5	68.1	15.5	0.8
Beef heart	2	16.6	73.5	8.7	0.8
Lamb heart	2	14.3	65.5	19.4	0.7
Pork heart	1	16.4	76.5	6.2	0.9
Pork heart	2	16.8	74.6	7.5	0.8
Beef brain	1	11.0	78.0	10.9	0.6
Beef brain	2	10.9	79.2	9.6	0.7
Lamb brain	2	11.8	79.3	9.2	0.8
Pork brain	1	11.4	78.5	9.9	0.7
Pork brain	2	11.2	78.4	9.9	0.7
Beef pancreas	1	12.9	61.2	24.4	1.0
Beef pancreas	2	15.1	75.2	10.0	0.8
Lamb pancreas	2	15.9	75.3	9.9	1.3
Pork pancreas	1	17.3	65.7	16.7	1.0
Pork pancreas	2	16.4	64.7	20.4	0.9
Beef lung	1	17.8	78.4	2.7	1.0
Beef lung	2	18.8	79.0	0.9	1.0
Lamb lung	2	17.9	78.8	1.5	1.1
Pork lung	1	14.0	82.8	2.6	0.8
Pork lung	2	11.7	85.9	1.8	0.8
Beef spleen	1	16.9	75.2	6.9	1.1
Beef spleen	2	18.4	77.8	2.6	1.5
Lamb spleen	2	18.6	77.7	3.1	1.3
Pork spleen	1	16.2	71.2	11.7	1.1
Pork spleen	2	17.3	79.3	1.0	0.9

[b] N × 6.25. The proximate analyses were made by the Service Laboratory, American Meat Institute Foundation.

lent agreement. A composite sample for 10 animals was used and this provides a much sounder basis for computing the vitamin content of similar samples than can be obtained from data on individual organs. More variation was noted in the niacin values for the 2 composite samples in certain instances (pork liver, beef and pork pancreas).

The vitamin content of the livers from older animals (see Table 2 and text) was similar to that for the livers from younger animals. Variations in vitamin potency appear to be more markedly influenced by the vitamin intake than by the age of the animal [see references cited elsewhere *(10)*].

TABLE 2

The thiamine, riboflavin and niacin content of various organ meats (all values expressed as mg./100 g. on a fresh basis)

Material	Source[c]	Thiamine	Riboflavin	Niacin
Beef liver	1	0.18	2.51	11.9
Beef liver	2	0.21	2.89	11.3
Lamb liver	2	0.29	3.90	12.1
Pork liver	1	0.25	2.97	18.1
Pork liver	2	0.25	3.01	9.8
Beef liver (cow)	1	0.27	3.17	16.0
Beef liver (cow)	2	0.23	2.85	12.3
Pork liver (sow)	1	0.26	3.03	17.4
Pork liver (sow)	2	0.19	2.76	11.1
Beef kidney	1	0.27	1.95	5.47
Beef kidney	2	0.29	1.82	5.19
Lamb kidney	2	0.38	2.18	6.77
Pork kidney	1	0.26	1.84	8.51
Pork kidney	2	0.26	1.88	8.68
Beef heart	1	0.23	0.77	6.61
Beef heart	2	0.24	0.90	6.53
Lamb heart	2	0.31	0.86	4.55
Pork heart	1	0.33	0.79	7.95
Pork heart	2	0.29	0.82	6.64
Beef brain	1	0.12	0.24	3.41
Beef brain	2	0.12	0.20	3.76
Lamb brain	2	0.15	0.26	3.71
Pork brain	1	0.16	0.28	4.17
Pork brain	2	0.15	0.27	4.38
Beef pancreas	1	0.12	0.31	2.47
Beef pancreas	2	0.15	0.37	3.75
Lamb pancreas	2	0.13	0.50	3.90
Pork pancreas	1	0.11	0.45	4.17
Pork pancreas	2	0.10	0.47	2.73
Beef lung	1	0.11	0.35	4.04
Beef lung	2	0.11	0.36	3.92
Lamb lung	2	0.11	0.47	4.66
Pork lung	1	0.10	0.30	3.47
Pork lung	2	0.07	0.24	3.22
Beef spleen	1	0.13	0.28	4.34
Beef spleen	2	0.13	0.28	4.13
Lamb spleen	2	0.09	0.27	4.66
Pork spleen	1	0.13	0.28	4.31
Pork spleen	2	0.13	0.32	4.29

[c] 1 and 2 designate two different companies from which the samples were purchased.

SUMMARY

The thiamine, riboflavin, and niacin content and the proximate composition of liver, kidney, heart, pancreas, spleen, lung, and brain from beef, lamb, and pork and liver from old cows and sows have been determined. Composite samples of the organs from 10 animals were prepared, and these composite samples were made from beef and pork carcasses obtained from 2 sources. Liver was found to contain 10 times as much riboflavin

and 3-5 times as much niacin as brain, lung, spleen or pancreas. The thiamine content of liver, kidney, and heart was approximately twice that of brain, spleen, pancreas, and lung.

The thiamine and riboflavin contents of the composite samples were in excellent agreement. More variation was noted in the niacin content of certain of the composite samples of the same organs.

No marked differences in the vitamin content of the organs from beef as compared to pork and lamb were noted. Livers obtained from old cows or from sows were similar in vitamin content to those obtained from younger animals.

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A STUDY OF CERTAIN FACTORS INFLUENCING THE JUICINESS OF MEAT[a,b]

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Experimentation has shown that tender cuts of beef, such as roasts and steaks, containing higher percentages of suet are juicier than lean cuts, as judged by a taste panel *(1, 2, 4)*. The conclusion, that beef with more fat is more juicy, would also be in accord with many of the studies of press fluid and total water content *(4, 5, 7, 12)*. However, Satorius and Child *(9)* have indicated no relationship between fat content and the amount of press fluid. In the recent work of Gaddis, Hankins, and Hiner *(4)*, it was found that both the scores for quality and for quantity of juice were influenced by the amount of intra-muscular fat and the fat content of the press fluid. These workers suggested that low intra-fat meat should yield more press fluid than that of high fat content if the meat were cooked with little moisture loss; if the meat were overcooked, the reverse would be true *(4)*. Thus, a high fat content coupled with low temperature cookery for short periods yields more juicy meat.

Little is known of the mechanism by which suet adds to the experience of juiciness. Whether the presence of intra-muscular fat induces a greater flow of saliva or a different mouth feeling or whether it has been associated previously with tender meat from which juice is more easily pressed are psychological factors which may be involved in the experience of meat juiciness. One suggestion has been made that suet changes the relative amount of juiciness because of differences in the rate of heat transfer *(11)*, and, therefore, differences in the degree of cooking. The fact that temperature and length of cooking affect the juiciness of beef roasts and steaks has long been established *(8)*.

Further work on the effect of suet on the juiciness of beef and on heat transfer is needed especially for less-tender cuts which require moist heat cookery. The interrelationships between the many factors which influence meat quality suggested the development of a small-sample method of braising and of determining cooking losses and relative juiciness wherein those factors could be investigated over a wide range of controlled conditions. Such a method has in the present study been applied in Experiment I to blended fat and lean beef under 3 sets of conditions and in Experiment II to uncovered and suet-covered lean meat. Studies on the rate of heat transfer and the melting characteristics of suet were included and determinations made of the vapor pressure of raw and cooked beef.

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EXPERIMENTAL METHODS

In Experiment I, blended samples of lean beef and suet were cooked in closed graduated centrifuge tubes, thus simulating braising. A split plot statistical design was used with the following variables: temperature of cooking: 70°, 80°, 90°, and 98°C.; length of cooking time: 5, 10, 20, and 30 min.; lean to suet proportion: 100% lean, 75% lean-25% suet, and 50% each of lean and suet. Three series were planned using this design. In the 6 replicates of Series I, the total weight of all samples was 5 g. heated in 15-ml. tubes. The samples of Series II (9 replications) contained 5 g. of lean plus the added amounts of suet. Series III was identical to Series I in the calculation of percentage of suet, but all samples weighed 10 instead of 5 g. Further, the 3 replicates of Series III were completed from a single cut of round steak. Series II and III samples were heated in 50-ml. tubes.

Round steak, Good grade, and kidney suet were purchased at random. The lean was coarsely ground in a food chopper, and the suet was cut into small pieces, then appropriate amounts were blended in a Waring Blendor to a uniformly pasty consistency. After the correct weight of blended meat mixture was squeezed from a pastry tube into previously weighed graduated centrifuge tubes, the tubes were stoppered with an aluminum-foil-covered cork and placed in a constant temperature water bath for cooking. At the end of the cooking period the cooking losses, juice (water phase) and fat loss in milliliters, were recorded and the tube and sample were inverted for draining, then reweighed.

In Experiment II, intact rectangular solids of semimembranosus muscle of beef, graded Good or Choice, were covered with a sheath of suet and compared to samples of lean only using as criteria, cooking losses and panel scoring. The smaller samples, 10 ± 0.5 g. in weight, were covered with an equal weight of thinly sliced suet, obtained from the inner portion of the bottom round. Thus, their total weight was equal to that of the lean meat which was cut into 20 ± 0.5 g. size. Heavy thread was used for holding the suet in place and also to suspend the samples in the 50-ml. tubes. The water bath was controlled at 85° ± 1.0°C., which temperature represents a simmering temperature in meat cookery and in preliminary tests was found to produce a *rare* stage after 10 min. of cooking, *medium* after 15 min., and *well-done* after 25 min. The tubes were placed in the bath at such intervals that all samples could be removed at one time. When the heating was completed, the threads and suet were removed from suet-covered samples and the larger lean pieces were cut into two parts, thereby making all portions of an equal size and shape when presented warm to the panel of 5 judges. Juiciness was judged on a 10-point score card. Ten replications were completed and the data analyzed statistically.

The rate of heat transfer in samples similar to those used in Experiment II was determined by placing a copper-constantin thermocouple through a rubber cork and into the center of each sample. Millivolt readings were made every 5 min. for 30 min. on a Leeds-Northup portable potentiometer; and then converted to degrees Centigrade. The results were plotted as a time-temperature curve.

The vapor pressure of the moisture in raw meat and meat heated at 85°C. for 10, 15, and 25 min. was determined by the procedure described by Johnson *(6)*. This isotenscope method was previously used on elastic gels by Briggs *(3)*. The samples for the vapor pressure study were prepared from semimembranosus muscle in the same manner and were of the same size as the lean beef samples used in Experiment II. The vapor pressure of each meat sample was divided by the vapor pressure of water determined for that day to obtain relative vapor pressure. The amount of water removed by each evacuation period was divided by the dry weight of each sample, converting water content into grams of water per gram of meat on a dry weight basis. Four replicates of duplicate samples were completed and the averages computed.

RESULTS

Experiment I. Results for the 3 series of blended lean and suet are recorded elsewhere in detail *(10)* in the form of 36 figures and 9 tables of statistical analyses. The average juice and total drip loss and loss of weight of only the samples of Series I heated at 80°C. are given in Figure 1 as an illustration. Likewise, the analysis of variance for Series I are presented in Table 1. For the sake of brevity, only a sum-

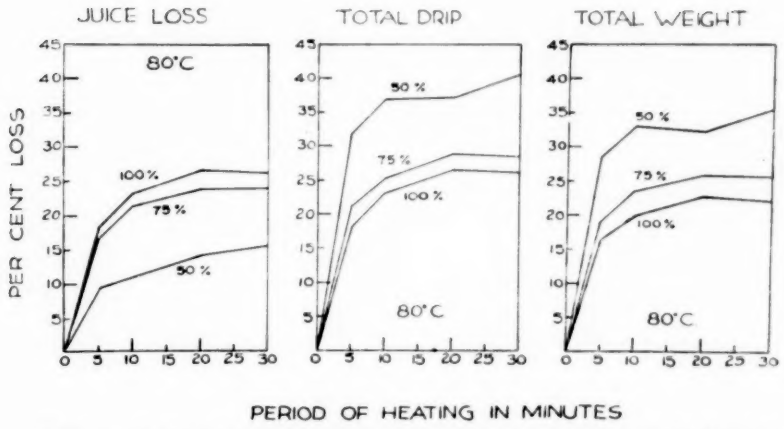

Figure 1. Average juice, total drip loss and total weight loss due to heating 5 g. blended lean and suet samples.

TABLE 1

Analysis of variance of cooking losses for 5 g. samples of blended lean and suet—Series I

Source of variation	Degrees of freedom	Mean squares for		
		Juice loss	Total drip loss	Weight loss
Among replications	5	413.28*	1.268	1.361*
Among temperatures	3	4024.51**	14.802**	13.534**
Replications × temperatures	15	97.59**	0.439**	0.375**
Among treatments	11	704.93	2.097	1.781
Among times	3	539.67*	1.738*	1.260**
Among ratios	2	3020.08**	8.917**	7.868**
Interaction	6	15.85	0.004	0.012
Replications × treatments	55	7.57	0.036	0.020
Replications × times	15	7.02	0.044	0.011
Replications × ratios	10	11.86	0.061	0.032
Replications × interaction	30	6.41	0.025	0.020
Temperatures × treatments	33	34.64**	0.145**	0.085**
Temperatures × times	9	103.15**	0.375**	0.144**
Temperatures × ratios	6	9.36	0.167**	0.197**
Temperatures × interaction	18	8.80	0.022	0.017
Residual	164	9.02	0.033	0.020
Total	286			

** Significant F-value at 1% level.
* Significant F-value at 5% level.

mary table of the significance of the sources of variation for all 3 series are given in Table 2.

The graphic presentation of juice loss for all series showed that increasing the proportion of suet decreased juice loss (water phase), whereas increasing the temperature of cooking and length of heating increased juice loss. There was, however, little difference in juice loss for the 75% and 100% lean samples at the lowest temperature, 70°C. Thus, beef cooked at low temperatures does not usually lose large quantities of juice. Total drip loss and total weight loss increased not only with temperature and length of cooking but also with increasing suet.

TABLE 2

Significance of sources of variations of cooking losses of blended suet and lean for all series of Experiment I

Source of variation	Juice loss			Total drip loss			Total weight loss		
Series	I	II	III	I	II	III	I	II	III
Among replications	*		**			**	*		**
Among temperatures	**	**	**	**	**	**	**	**	**
Replications × temperatures	**	**		**	**	**	**	**	
Among treatments									
Among times	*	**	**	*	**	**	**	**	**
Among ratios	**	**	**	**	**	**	**	**	**
Interaction			**						
Replications × treatments					*				
Replications × times									
Replications × ratios		**	*		**	**		*	
Interaction									
Temperatures × treatments	**	**		**	**	*	**	**	
Temperatures × times	**	**		**	**	**	**	**	
Temperatures × ratios		**		**	*		**	*	**
Interaction					*			*	

** Significant F-value at 1% level.
* Significant F-value at 5% level.

The analyses of variance for juice, total drip, and total weight loss showed highly significant or significant differences for the main factors of *temperature*, *length of cooking time*, and *suet-lean ratio* in all series, Table 2. It is of interest to note that in Series III, even though only 3 replicates were completed and these differed significantly, similar statistical significance for these main factors was obtained. This illustrates the small degree of replication needed and the rapidity of accumulating data in this small-sample braising method. However, since only one cut of round steak was used the data are more limited in application than are Series I and II. There is to be noted the greater number of significant two-factor interactions in Series II. Since this series had 9 replications, smaller differences may have been detectable. On the other hand, fewer significant interactions may have occurred in Series III because of the larger, constant volume and composition of samples which would tend to eliminate variation in cooking losses due to differences in the rates of heat penetration.

Experiment II. The results for lean and suet-covered samples, Table 3, showed that increasing the length of cooking increased juice loss and decreased judges' scores and that the covering of suet decreased juice loss. The analysis of variance of judges' scores for juiciness showed a highly significant decrease in juiciness as the samples were cooked from the rare to the well-done stage, which was as might be expected from the results of the objective methods of Experiment I. However, the judges were unable to detect a significant difference in juiciness between suet-covered and lean samples. Since the analysis showed that the scores were consistent between replications and between judges, the panel may have been judging some factor or factors of juiciness in addition to juice loss. The regression coefficient of average judges' scores on average juice loss was highly significant, —0.85; however the major decrease in judges' scores was due to time effect and not suet effect.

In the study of the rate of heat transfer in similar suet-covered and lean samples, Figure 2, each increment of temperature resulted in a more rapid rate of heat transfer in both lean and suet-covered samples. It was found that the rate of heat penetration was retarded by the covering of suet and explains the results shown in Table 2, when samples were heated at 85°C. Since heat transfer was slower in the suet-covered samples cooked 10 and 15 min., some of the differences in juice loss were probably due to a lesser degree of cooking. The internal temperature of both types of samples after heating for 25 min. at 85°C. were within one degree of one another and the difference between them in juice loss less than those heated for 10 or 15 min.

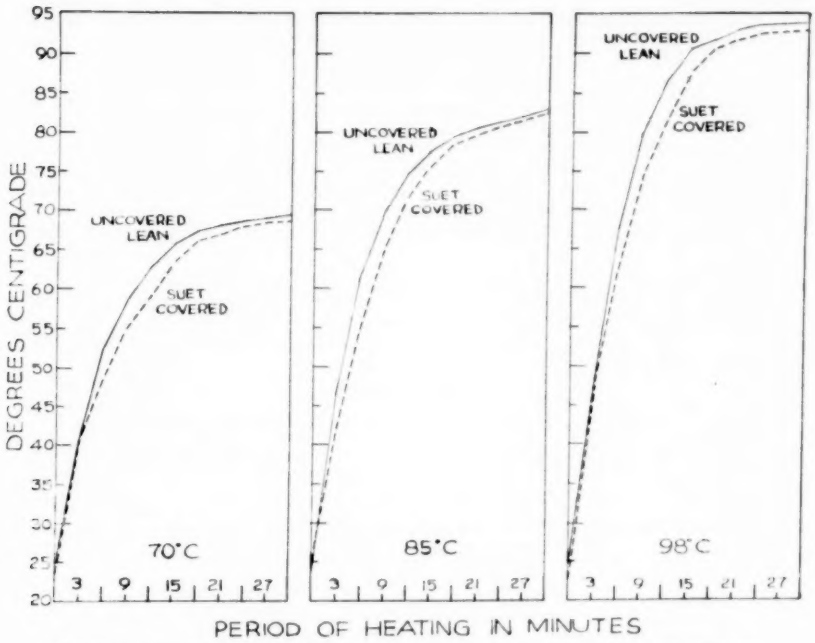

Figure 2. Average rates of heat transfer in suet-covered and lean beef samples.

TABLE 3

Suet-covered versus lean beef samples: averages and analysis of variance of judges' scores for juiciness

Length of cooking time	10 min.		15 min.		25 min.	
Description	Suet-covered	Lean	Suet-covered	Lean	Suet-covered	Lean
Percent juice loss	8.7	17.3	22.7	31.1	32.6	35.8
Judges' scores for juiciness	7.8	8.0	5.6	5.4	4.4	4.0

Source of variation	Degrees of freedom	Mean square
Replications	9	2.68
Treatments	5	146.06**
Suets	1	1.47
Time of cooking	2	361.83**
Interaction	2	2.59
Judges	4	1.51
Replications × treatments	45	4.35**
Replications × judges	36	2.33
Treatments × judges	20	1.97
Suets × judges	4	2.41
Time × judges	8	2.10
Suets × time × judges	8	1.63
Error	180	2.49
Total	299	

** Significant F-value at 1% level.
* Significant F-value at 5% level.

Since the characteristics of suet appeared to enter into the heat transfer studies, the melting point of suet was determined. In a capillary tube it melted at 48°C., but when kidney suet was forced into the lower arm of a ⅛-in. diameter glass U-tube and heated, the suet became translucent at 45°C., became liquid at 46.5°C., and drained at 53°C. When these same samples were solidified and a slight suction applied during heating, the sample was pulled free at 39°C., indicating that some softening of the fat on the surface had occurred.

For further information on the nature of suet, internal temperatures were taken every 5 min. for 65 min. during the heating of rendered suet, blended suet, intact suet, and connective tissue. Approximately the same rectangular volumes of each material were heated in a 325°F.(163°C.) oven. The rendered suet, Figure 3, heated most rapidly

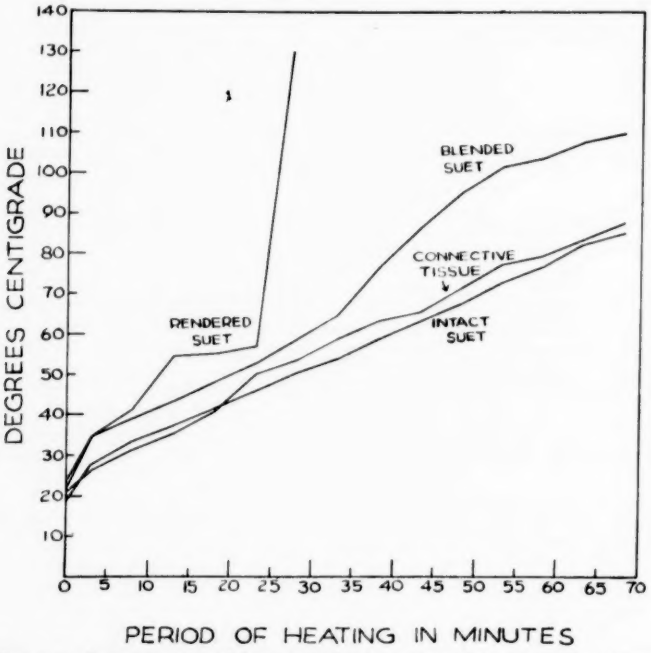

Figure 3. Average rates of heat transfer in suets and in connective tissue.

indicating that the presence of connective tissue decreased the rate of heat transfer in suet. Intact strands of connective tissue were more inhibitory to heat transfer than minced connective tissue, since intact suet and connective tissue heated at approximately the same rate.

Another approach to the problem of juiciness in meat was the study of relative vapor pressures in raw beef and in that heated at 85°C. for 10, 15, and 25 minutes. These data are presented in Table 4. As the meat was cooked, the relative vapor pressure decreased. Since water in the "bound" form cannot exert a vapor pressure, this result was taken to mean that the amount of "bound water" increased as cooking progressed. The exact amount of "bound water" was not determined as such by this method. However, since the samples exerted a vapor pressure even after the second 30-min. evacuation period, and "bound water" does not exert a vapor pressure, it can be seen from Table 4 that only a small amount of the water in either raw or cooked beef is in the "bound" form. It was assumed that after evacuation overnight all of

TABLE 4

Water loss and relative vapor pressures (R.V.P.) of raw and heated beef

Evacuation periods	Raw		Cooked 10 min.		Cooked 15 min.		Cooked 25 min.	
	g. H_2O*	R.V.P.	g. H_2O*	R.V.P.	g. H_2O*	R.V.P.	g. H_2O*	R.V.P.
1 min.	0.28	1.01	0.26	0.99	0.20	0.94	0.18	0.92
30 min.	1.64	0.95	1.46	0.95	1.23	0.88	1.11	0.87
Additional 30 min.	2.20	0.87	1.88	0.76	1.55	0.62	1.41	0.64
Overnight	2.79	0.08	2.04	0.06	1.71	0.07	1.55	0.06

* Based on dry weight basis computed from data of overnight evacuation period.

the water was removed from the sample; these values were, therefore, considered to be the total water content in the various samples. The percentage of total water in the original raw semimembranosus muscle used in these studies averaged 73.3%.

DISCUSSION

The heat transfer studies showed that probably the major part of the juice retention caused by suet content was due to slower rates of heat transfer in the suet and connective tissue. However, heat transfer did not appear to be the entire solution of the problem. Many factors are involved in the rate of heat transfer: the amount of exposed surface, the difference in volume after heating for a time, the heat of fusion of fat, its rate of conductivity, as well as factors involved in the protein components. Heat transfer results are dependent upon these factors and their interrelationships and therefore it is difficult to isolate the factors which have the greater influence.

In addition to differences in the rate of heat transfer and suet content, juiciness in beef is influenced greatly by length and temperature of cooking. One other factor was inadvertently shown on 20-g. samples of semimembranosus muscle heated at 85°C. When the juice loss was recorded immediately after the cooking periods, the values were 19%, 26%, and 34.5% for heating periods of 10, 15, and 25 min., respectively. However, some juice was forced out during the cooling period and brought the total loss to 26.4, 29.7, and 35.3%. Thus for the 10-minute samples, there was a better correlation between the weight loss and juice loss for cold beef than for hot or warm, whereas for the longer cooked samples, greater juice loss had already occurred during heating. These observations further explain why total drip loss, measured immediately after cooking, and total weight loss, measured after draining, were not identical in the figures and analyses of Experiment I.

The blended samples of Experiment I have their counterpart, as far as lean to suet ratios are concerned, in mixtures used for patties and meat balls. In the usual cookery procedures, however, meat patties are not braised and meat balls usually have ingredients such as bread crumbs, cereal, starch or flour to absorb some of the juice exuded during heating. However, the general principles of low-temperature- and short-time-cooking recommended for tender cuts of meat are shown to apply also to braised samples of fat and lean mixtures. It is hoped to elucidate in further work, using the scheme of small samples of beef braised in test tubes, some of the conditions applicable to pot roasts.

SUMMARY

A small-sample braising technique has been described whereby drip losses from beef containing known added quantities of suet has been accurately measured under good statistical control.

Loss of the water phase of juice from blended samples of lean and suet was significantly decreased with increasing percentages of suet. Increasing temperature of braising and length of cooking time significantly increased juice loss. Suet-covered samples of lean gave similar results. Judges' scores for juiciness decreased significantly with increasing juice loss due to longer cooking, but the judges did not detect the lower juice losses in suet-covered samples. The regression coefficient for average percent juice loss on average judges' scores was —0.85, which was highly significant. The rate of heat transfer was found to be slower in the braised suet-covered samples than in those entirely of lean.

Connective tissue decreased the rate of heat transfer in solid blocks of suet more than in samples where the connective tissue had been minced or removed. The rate of heat penetration in equal volumes of connective tissue and intact kidney suet was approximately the same. The melting of suet was found to be a gradual process, softening at 39°C., and with drainage of liquid fat at 53°C. The approximate melting point was found to be 48°C.

A preliminary study on the vapor pressure of the moisture in beef indicated that more water is "bound" as the meat is cooked, but that the quantity of "bound water" as judged from these vapor pressure determinations was comparatively small for either raw or cooked beef. Total water content for raw semimembranosus beef muscle was found to be 73.3%.

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POST-MORTEM CHANGES IN STORED MEATS. V. EFFECT OF CARBON DIOXIDE ON MICROBIAL GROWTH ON STORED FRANKFURTERS AND CHARACTERISTICS OF SOME MICROORGANISMS ISOLATED FROM THEM[a,b]

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Published information concerning the microorganisms causing spoilage of cured meats is rather meager. Roderick and Norton *(9)* found micrococci to be a cause of slime on frankfurters and studied a large number of isolates in some detail. These strains were aerobic, rarely proteolytic and largely non-pigmented. Most of the strains fermented one or more sugars, almost always without gas production. Rather high salt concentrations were tolerated, and these organisms were destroyed on the sausages by the usual cooking processes.

Yellow micrococci and several types of bacilli and lactobacilli were isolated by Ingram *(3)* from slimes on bacon. On nutrient agar containing salt, Garrard and Lochhead *(2)* obtained Gram-negative cocci or coccoid rods from fresh pork sides. They stated that these were similar to bacteria found by Landerkin (unpublished work) to be the most numerous bacteria on 5% salt agar plates from slimy bacon. These unidentified bacteria were of interest because of their pronounced lipolytic activity.

From slimy sausage, Kuhl *(4)* found a white yeast in practically pure culture. The fermenting power of this yeast was unusually slight. Mrak and Bonar *(5)* discovered that the pastry slime on several samples of wiener sausage was composed of a mixture of yeasts and bacteria. All of the isolates of yeasts were similar and were considered to be *Debaryomyces Guilliermondii* var. *nova Zeelandicus* Lodder. This yeast tolerated high concentrations of salt, was weakly fermentative, and multiplied on sausage at temperatures as low as 39°F.(4°C.).

Yesair *(11)* obtained the mold genera *Penicillium, Aspergillus, Mucor, Rhizopus, Alternaria, Monascus,* and *Monilia* from sausages.

Niven *et al. (6)* found that greenish discolorations on the surfaces of frankfurters ordinarily were due to types of *Lactobacillus* and *Leuconostoc.*

Very little has been reported concerning the action of carbon dioxide on the microbial flora of cured meats. According to Coyne *(1)*, micrococci were among the bacteria which were strongly inhibited by carbon dioxide at low temperatures, and Tomkins *(10)* observed that 30% carbon dioxide almost completely prevented the growth of most meat-attacking molds on an artificial nutrient medium.

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[a]Appreciation is expressed to the Pure Carbonic Company, a division of Air Reduction Co., Inc., New York, N. Y., for support, in part, of this research.

[b]Journal Paper No. J-2123 of the Iowa Agricultural Experiment Station, Ames, Iowa. Project 1161.

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In a previous paper *(7)* carbon dioxide was found to extend storage life of frankfurters. The effectiveness of the gas was enhanced by a reduction in temperature and also by an increase in concentration up to about 50%. With higher levels, no improvement of keeping time was obtained; the onset of spoilage was indefinite and characterized by a thin, watery slime on the surface of the sample and a noticeable increase in acidity which was detected both by taste and by pH measurement.

In order to extend the earlier findings on the storage of frankfurters, a study was made of the types of microorganisms responsible for spoilage and of their growth characteristics under various conditions.

EXPERIMENTAL

All-meat, skinless frankfurters were stored under refrigeration in glass containers with various concentrations of carbon dioxide. A detailed description of the storage methods used has been given previously *(7, 8)*. At various times during storage, samples were removed for examination and bacterial counts.

For microbial counts on frankfurter surfaces, 10 g. of screened, washed sea sand and enough distilled water to give a total volume of 100 ml. were added to each of a number of 500-ml. wide-mouthed bottles equipped with solid rubber stoppers. Bottles and contents were sterilized by autoclaving. An entire frankfurter was placed in a bottle which was then shaken 200 times. From the resulting suspension of microorganisms, aliquots of suitable dilutions were used in preparing agar pour plates. Nutrient agar with 1% salt and 0.5% yeast extract added was used in obtaining counts of micrococci and total counts. Tomato juice agar (Difco) with 0.3% additional agar and 0.5% yeast extract was employed in counting lactobacilli, yeasts, and molds. Plates were incubated 4 days at 68°F.(20°C.) before counting. The several different kinds of organisms found on the agar plates were differentiated by differences in colony size and appearance. To check accuracy of visual observations, several colonies of each type from every plate counted were used in preparing Gram stains for microscopic examination. Colonies were picked for further study from plates representing different samples and storage conditions.

RESULTS

The bacteria originally present on frankfurters were predominantly Gram-positive types. Micrococci, bacilli, and sarcinae were most numerous, while lactobacilli and Gram-negative bacteria were present in smaller numbers. Appreciable numbers of yeasts sometimes were encountered; molds, while probably present in all cases, were comparatively few in number.

Initial counts on frankfurters showed a rather remarkable consistency. About three-quarters of the counts made on fresh samples over a period of 2 years fell within the range 200% to 50% of the average count of 4,120 per frankfurter. Counts made on meat from the interior of fresh samples averaged 5,800 per gram, all of these apparently being spore-formers.

It was learned early in this work that simple plate counts were not suitable for demonstrating the effect of carbon dioxide on microbial changes. A series of plate counts were made on frankfurters stored with 0, 5, 15, 25, 50, 75, and 96% carbon dioxide at 40°F.(4.4°C.). The resulting growth curves are shown in Figure 1. These are total counts (exclusive of molds) from nutrient agar plates. Counts made on samples stored with 25% carbon dioxide were too erratic to permit construction of a curve. The results of this study were quite surprising in view of the effectiveness of carbon dioxide in prolonging storage life. Increasing the gas concentration had comparatively little effect upon total numbers present after a given time, and no consistent relationship could be demonstrated between keeping time and total count.

Since the type of flora prevailing on stored frankfurters seemed to depend largely upon the carbon dioxide concentration used, a study of the individual types of microorganisms was undertaken. The order in which various kinds of microbiological deterioration usually became visible is listed for a number of gas levels in Table 1.

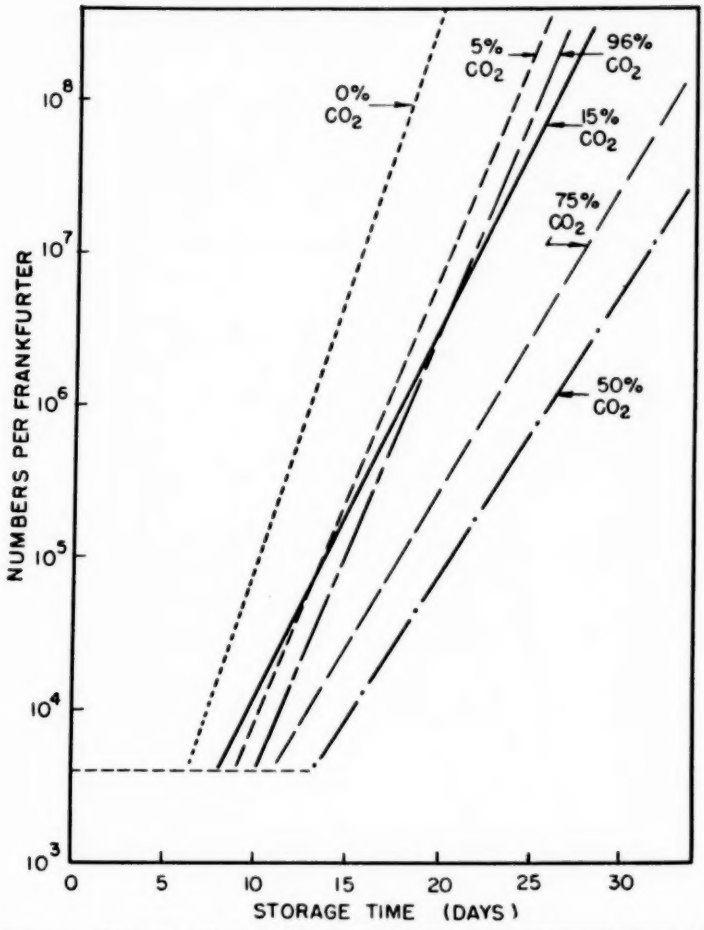

Figure 1. Effect of carbon dioxide on growth curves of microorganisms on frankfurters stored at 40°F.(4.4°C.).

TABLE 1

Effect of carbon dioxide on the type of microbial spoilage of frankfurters

CO_2 level (% by volume)	Type of spoilage (listed in usual order of appearance)
0	Mold colonies, micrococcus and yeast colonies or slimes, greening.
5	Mold colonies, micrococcus and yeast colonies or slimes, greening.
15	Micrococcus and yeast colonies or slimes, mold colonies, greening.
25	Micrococcus and yeast colonies or slimes, greening, mold colonies.
50	Thin colonies or watery slime of lactobacilli, greening, micrococcus colonies, yeast colonies, mold colonies.
75	Lactobacillus colonies and slimes, micrococcus colonies.
96	Lactobacillus colonies and slimes.

The individual development of micrococci, yeasts, molds, and lactobacilli was followed by means of plate counts made at suitable intervals from frankfurters stored at 45°F.(7.2°C.) at carbon dioxide levels of 0, 5, 15, 25, 50, 75, and 96%. Figure 2 shows growth curves obtained for micrococci. As carbon dioxide concentration increased, logarithmic growth rates decreased and lag time was extended. The decline in numbers with 96% may have been partially or mainly due to lack of oxygen.

Yeast counts are given in Figure 3. Yeast counts tended to be erratic and frequently no yeast colonies were found in the initial plates. For Figure 3, an initial count of 10 yeasts per frankfurter was arbitrarily selected as the starting point for

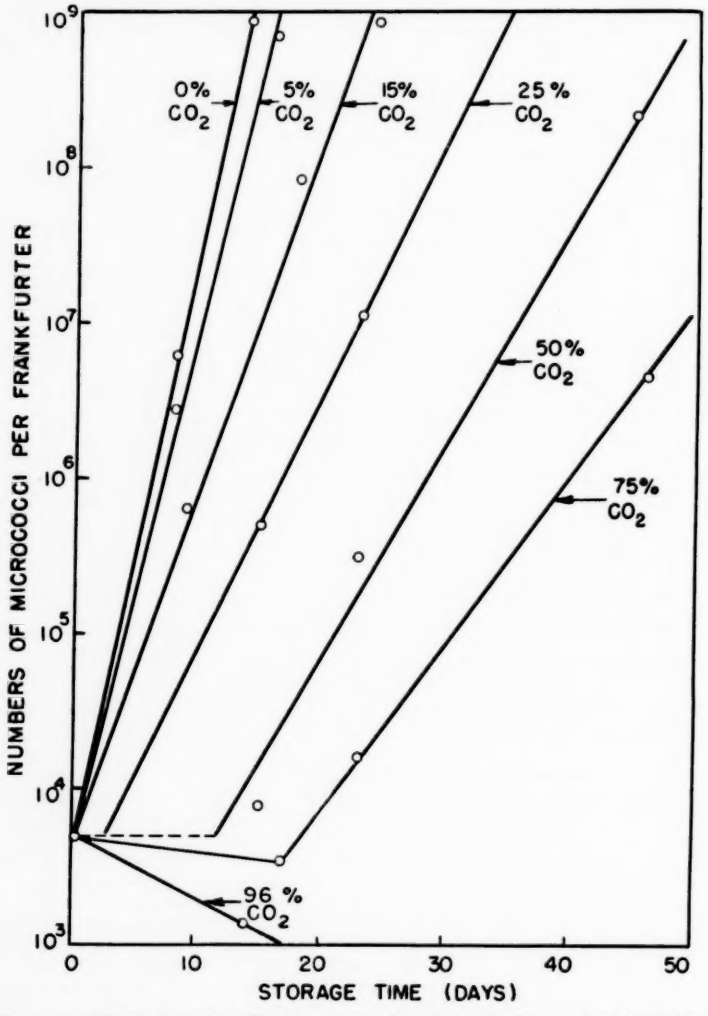

Figure 2. Effect of carbon dioxide on growth curves of micrococci on frankfurters stored at 45°F.(7.2°C.).

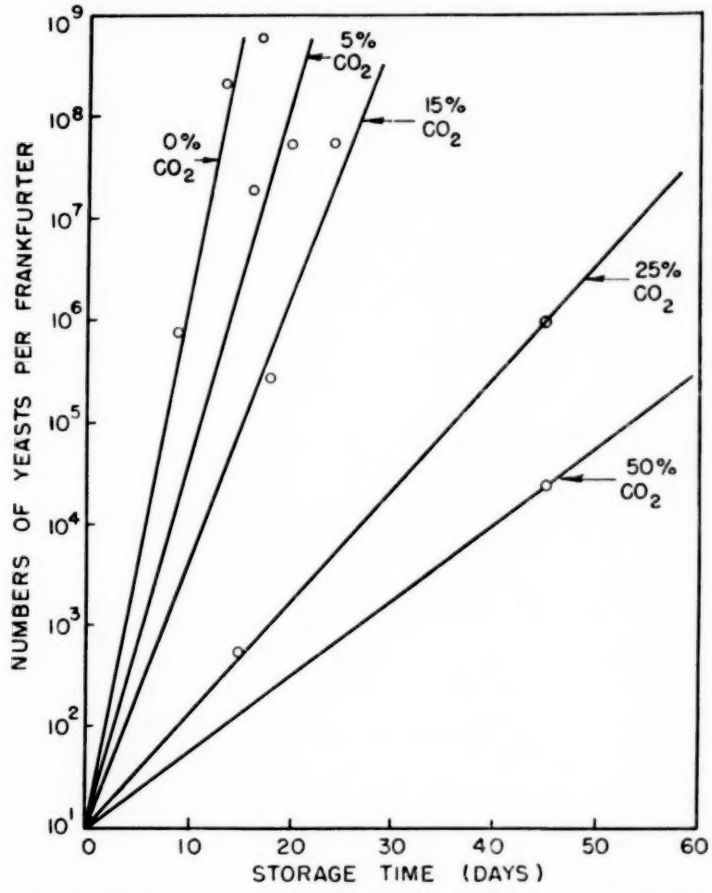

Figure 3. Effect of carbon dioxide on the growth of yeasts on frankfurters stored at 45°F.(7.2°C.).

the various curves. Although not true growth curves, the straight lines in the figure serve to indicate the relative effectiveness of different carbon dioxide levels in inhibiting yeast growth. Growth became progressively slower as gas level was increased; no yeasts were found during 45 days' storage with 75 and 96%.

It was not considered practicable to represent mold increase by a regular curve based on plate counts. Mold growth was observed on the control plates at 2 weeks, but was not noticed at 5, 15, and 25% carbon dioxide until 16, 23, and 45 days, respectively. No evidence of mold development could be seen at any time when carbon dioxide levels of 50% or greater were used. These results, along with visual observations, showed that molds could be controlled at carbon dioxide levels of 15% or higher and probably could be disregarded entirely at levels of 50% or greater.

Counts made on lactobacilli are represented by the growth curves shown in Figure 4. There is no curve for 0% carbon dioxide because, in that case, other faster growing organisms obscured lactobacillus colonies in the plates. Curves for 15 and

25% were so nearly identical that they were combined in the figure; this applies also for the 50, 75, and 96% levels. Lactobacilli were not influenced to any great extent by the presence of carbon dioxide. Some restriction of growth was indicated up to the 50% level, but no appreciable advantage resulted from higher concentrations. Such differences as were attained appeared to depend as much or more on extension of lag time as on an increase in generation time during logarithmic reproduction.

No study was made of yeasts from frankfurters. Molds were identified as to genus only. Nearly all of the molds isolated from stored frankfurters were found to belong to the genus *Penicillium*. Other genera found occasionally were *Sporotrichum*, *Zygorhinchus*, *Monilia*, *Mucor*, *Aspergillus*, and *Alternaria*. An increase in the relative num-

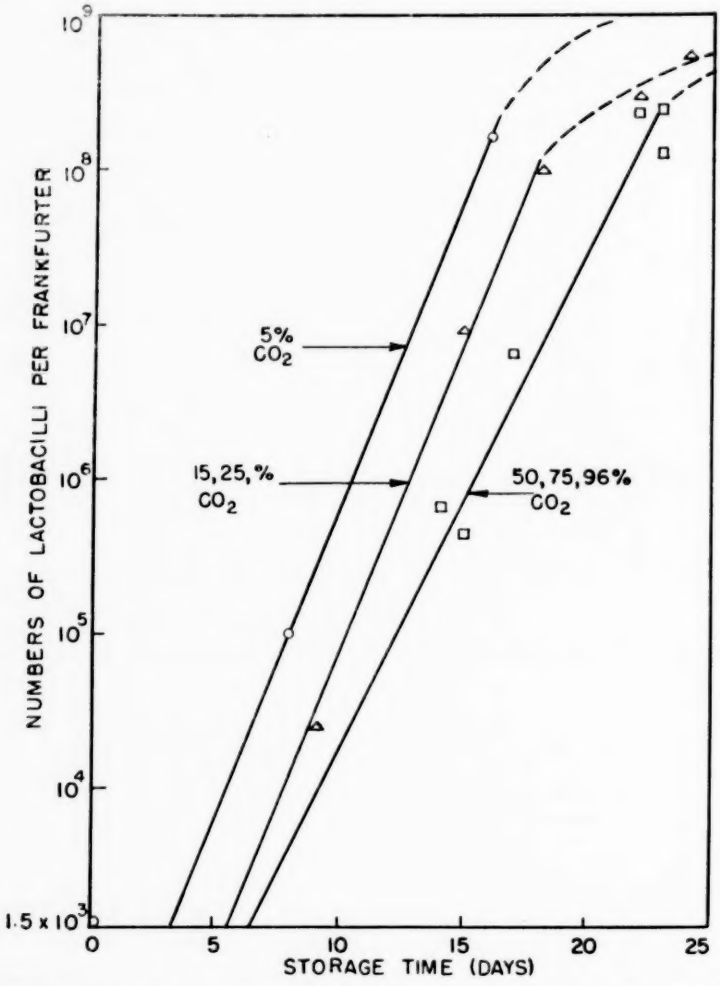

Figure 4. Effect of carbon dioxide on growth curves of lactobacilli on frankfurters stored at 45°F.(7.2°C.).

TABLE 2

Characteristics of types of micrococcus isolated from stored frankfurters

Type	Chromogenesis	Nitrates reduced	Ammon. phosphate used as source of nitrogen	Urea used as nitrogen source	Gelatin liquefied	Litmus milk	Acid from glucose	Acid from glycerol	Acid from manitol	Fat hydrolyzed	Number of cultures
1	Yellow	−	+	+	−	Alkaline	+	−	−	−	1
2	Yellow	+	+	−	+	Acid, reduced, some coagulation	+	+	+	+	2
3	Pink	+	+	+	+	Alkaline	−	−	−	+	1
4	White, buff	−	+	−	−	Alkaline	+	+	−	−	3
5	Buff	−	+	+	+	Acid, may be peptonized	+, −	+, −	+, −	+	8
6	White	−	−	−	−	Peptonized	+, −	−	−	+	6
7	White	−	−	−	−	No change or alkaline	−	−	−	+	5
8	White	+	+	+	+	Peptonized	+	+	−	+, −	6
9	White	+	+	+, −	+	Usually red., may be sl. acid or sl. alk.	+	+	+	+, −	11
10	White, buff	+	+	+	−	Acid, no coagulation	+	+, −	−	+	4
11	White	+	+	+	−	Acid, some coagulation	−	−	−	+	2
12	White	+	−	−	+	Acid, may coagulate	+	+	−	−	6
13	White	+	−	−	−	Acid	−	−	−	+	1
14	Buff	+	−	−	−	Alkaline	+	−	−	−	1

bers of *Monilia* was noted at the lower storage temperatures (30° and 35°F.; −1.1° and 1.7°C.).

Members of the genus *Bacillus* were found frequently on the surface and always in the interior of fresh frankfurters. However, these organisms played little part in spoilage of samples at refrigerator temperatures below 50°F.(10°C.). At 50°F. large numbers of aerobic sporeformers were obtained on several occasions from the interior of frankfurters stored without carbon dioxide.

None of the samples stored at temperatures of 50°F.(10°C.) and below showed evidences of putrefaction. Apparently sporulating anaerobes cause no difficulties at such temperatures.

A detailed study was made of 57 cultures of Gram-positive micrococci isolated from stored frankfurters which had reached or were approaching the slimy stage. Cultures were divided into 14 types on the bases of pigmentation, nitrate reduction, utilization of $(NH_4)H_2PO_4$ as sole source of nitrogen, gelatin liquefaction, fermentation of glucose, and action on litmus milk. A description of each type and the number of cultures in each are shown in Table 2. None of the types could be identified definitely with any of the species of *Micrococcus* described in Bergey's Manual of Determinative Bacteriology (6th ed.). Types 8 and 9, which contained 30% of the cultures, resembled the description given for *Micrococcus cascolyticus* in many respects.

The results differ from those of Roderick and Norton *(9)* in that most cultures isolated in this work liquefied gelatin and quite a few peptonized milk. Results are similar in that most cultures produced white colonies on agar and none produced gas from sugars.

Another group whose classification was not clear was included with the Gram-positive cocci. This group was composed of Gram-negative cocci or coccobacilli usually occurring in pairs. Microscopic examination also revealed sticky conglomerations of cells in most cases and occasionally tetrads. These organisms were frequently found in large numbers together with Gram-positive micrococci on nutrient agar plates made from stored frankfurters and were similarly affected by carbon dioxide. Surface colonies on agar were a greyish white color, convex, and butyrous in consisteney. No cultures liquefied gelatin or produced acid from glycerol; ability to hydrolyze fat varied widely. Some other physiological characteristics of the 15 cultures studied are listed in Table 3.

TABLE 3

Physiological characteristics of gram-negative bacteria isolated from stored frankfurters

Number of cultures	Nitrates reduced	Gelatin liquefied	Reactions in litmus milk	Acid from glucose[a]
2	−	−	Sl. alkaline	−
3	−	−	Peptonized	+
9	+	−	Acid, little or no coag.	+
1	+	−	Sl. alkaline	−

[a] No cultures produced gas from glucose.

Of 120 cultures of lactobacilli isolated, all produced acid from glucose but none gave gas in the Durham fermentation tubes employed. Only 2 cultures produced visible greening on the cut surfaces of steamed pieces of frankfurter, and it was impossible to repeat these results at a later trial. Individual strains were not studied in detail. Morphologically, organisms varied from long thin rods with rounded ends to small, short rods with square ends. All were Gram-positive in young cultures; chains were usually present. In nutrient agar plates colonies were subsurface and very small. In tomato juice agar, subsurface colonies were larger, usually 1 to 2 mm. in diameter, white, and lens shaped or angular. In addition, tiny feathery colonies, barely visible to the naked eye, were frequently encountered. In several cases lactobacilli were found growing in small (1 to 3 mm.), raised surface colonies on tomato juice agar.

DISCUSSION

From the preceding results a reasonably clear picture of the microbiological spoilage of frankfurters can be drawn. With storage in air, spoilage is due to molds, micrococci, and yeasts. Which of these is the primary agent will be determined by such factors as initial contamination, relative humidity, and, to some extent, temperature. This study and information obtained from workers in the meat industry indicate that molds normally are the greatest trouble makers. However, there is ample evidence that, under some conditions, micrococci *(9)*, yeasts *(4, 5)* and members of the family *Lactobacteriaceae (6)* can cause spoilage outbreaks of great economic importance.

Application of carbon dioxide to storage atmospheres changes the picture markedly. Mold growth is considerably restricted by moderate concentrations (15 to 25%) and is appreciably reduced by 5%, while high levels prevent any important mold development. Growth of micrococci and yeasts is progressively reduced as carbon dioxide concentration is increased; reproduction proceeds very slowly at the higher percentages. Lactobacilli, on the other hand, are influenced much less by the gas. Use of levels of more than 50% seems to be of no value in retarding proliferation of these bacteria; indeed, there is evidence (Fig. 1) that very high concentrations may sometimes permit more rapid growth than lower levels. Any influence of carbon dioxide in retarding development probably is opposed by the tendency of low oxygen tensions accompanying high gas levels to promote growth of the microaerophillic lactobacilli while restricting other organisms. At any rate, the surface flora found with high carbon dioxide percentages (50 to 96) is composed almost entirely of lactic acid organisms. In the range 50 to 96%, the failure of changes in the gas concentration to affect substantially the growth rate of these bacteria explains the constancy of the keeping times found in this range.

The term "lactobacilli" has been used rather inclusively in this work to refer to a group of bacteria which may contain several members of the family *Lactobacteriaceae*. It is possible that some of the organisms isolated may belong to the genus *Leuconostoc*, although this was not indicated by morphological characteristics. Some cultures grew fairly well aerobically and these, perhaps, would fall into the genus *Microbacterium*.

The vast majority of the cultures of lactobacilli isolated are different from the organisms shown by Niven *et al. (6)* to produce greening on sausages (as evidenced by inability to green surfaces of steamed frankfurters). However, the development of green discolorations on some of the frankfurter samples showed that the latter organisms also were present. It is interesting that greening never occurred with samples stored under 75 to 96% carbon dioxide although it did appear occasionally with replicates subjected to lower gas levels. Lack of oxygen in the storage container might have prevented completion of the greening reactions; however, in that case the color should have appeared upon subsequent exposure to air. This was not the case. Perhaps, then, carbon dioxide is more effective against the greening lactic acid bacteria than against some other types.

With the high carbon dioxide levels, spoilage does not coincide with the attainment of maximum counts. Numbers of lactobacilli reach very

high values some time before deterioration is evidenced by marked changes in appearance, flavor, or pH. It appears that, after maximum numbers are obtained, an additional period of metabolic activity is required for the production of definite changes in the characteristics of the frankfurter.

SUMMARY

Carbon dioxide in the atmosphere surrounding stored frankfurters not only influenced the growth of microorganisms but also had a selective action on the types of organisms developing on the frankfurter surface and ultimately causing spoilage. Growth of molds, micrococci, and yeasts was more effectively retarded as the gas level was raised. Lactic acid bacteria, on the other hand, were only slightly affected by carbon dioxide and had little competition from other organisms at gas concentrations above 50%. The net result of the action of carbon dioxide on the flora of the frankfurter was improvement of the storage life of this product. This effect was due not so much to control of the total microbial population as to restriction of the types which most rapidly cause deterioration.

The mold genus commonly found was *Penicillium*. No importance could be attached to the growth of aerobic or anaerobic spore-forming bacteria at refrigeration temperatures. Attempts to identify slime-forming micrococci with known species were unsuccessful although many of the cultures were similar to *Micrococcus caseolyticus*. Isolates of lactic acid bacteria were not capable of causing green discolorations on sausages.

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ROASTING TIME AND TEMPERATURE REQUIRED TO KILL FOOD POISONING MICROORGANISMS INTRODUCED EXPERIMENTALLY INTO STUFFING IN TURKEYS

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Bacterial food poisoning from poultry constitutes a considerable percentage of the total food poisoning outbreaks. Feig *(7)* stated that poultry was the vehicle of streptococcal infection "twice as frequently as meat, bakery products and potatoes." Poultry was further involved in 22% of 304 staphylococcic outbreaks and in 18.5% of 54 salmonella outbreaks. Reports of the Federal Security Agency *(6)* listed 15 outbreaks for 1950, with 558 cases in which turkey was considered the vehicle of infection. For the period from February 14, 1951, to June 7, 1952, a total of 13 outbreaks, with 661 cases, were noted. The number of outbreaks was undoubtedly greater than thus recorded, for many are not officially reported *(2)*. In view of the role played by poultry as a cause of food poisoning, it was considered of interest to ascertain (a) the temperature required to kill streptococci (enterococci), staphylococci, and salmonellae in the interior portions of stuffed turkey, (b) the several factors which may affect the successful attainment of this lethal temperature and (c) the effectiveness of currently recommended roasting procedures with respect to the achievement of this lethal temperature.

MATERIALS AND METHODS

Cultures. The relative heat tolerance of *Streptococcus faecalis* R5, R7, R and of *Streptococcus liquefaciens* A and R26 was determined by dispensing one drop of 24-hour cultures in duplicate 16-mm. tubes containing 7 ml. of a tryptone-yeast extract-glucose-dipotassium phosphate broth and heating in a thermostatically controlled water bath. After inoculation of the tubes the protruding portions of the cotton plugs were snipped off and a rubber stopper inserted. The rack of tubes was then immersed in a preliminary water bath at a temperature of about 122°F.(50°C.) for several minutes until the temperature in the culture tubes reached about 104°F.(40°C.). The rack was then transferred to the test water bath, the tubes being immersed to within 1 inch of the top. About 60-75 seconds elapsed before the attainment of the test temperature as determined by a thermometer in a control tube. The test temperature plus or minus 0.9°F.(0.5°C.) was maintained for a desired time period, after which the tubes were immersed in an ice bath. The rubber stoppers were removed, and the heat shocked culture tubes were incubated at 86°F.(30°C.) for 7 days. Under these experimental conditions none of the above streptococci survived 158°F.(70°C.) for ten minutes. The more heat tolerant strains R5, R7, and R survived 149°F.(65°C.) for 30 but not 60 minutes.

Similar heat tolerance studies on *Staphylococcus aureus* 210, 196, 161, S-6, and 184 revealed that none survived 149°F.(65°C.) for 5 minutes. The more heat tolerant strains 196 and 184 survived 140°F.(60°C.) for 15 but not 30 minutes.

Streptococcus faecalis R5, *Staphylococcus aureus* 210 and 196, and *Salmonella pullorum* 3239 were selected as the test organisms. *Streptococcus faecalis* R5 was originally isolated from turkey stuffing incriminated in a food poisoning outbreak *(3)*. The 2 staphylococci were coagulase positive and were established food poisoning strains as determined by monkey feeding tests *(5)*.

Media. I. The following plating media were used:

(a) For total counts—1% tryptone, 1% yeast extract, 1% glucose, 0.5% dipotassium phosphate, and 1.5% agar, pH 7.0 before sterilization.
(b) For streptococci—0.01% sodium azide and 0.1 unit of penicillin G per ml. were added to (a) before sterilization *(3)*.
(c) For staphylococci—100 g. of sodium chloride were added to 1,000 ml. of (a) before sterilization. Chapman Stone Medium and Staphylococcus Medium No. 110 were also used to a limited extent.
(d) For salmonellae—Endo Agar.

II. The following selective broths in 10-ml. quantities were used to determine the qualitative survival of the organisms in roasted stuffing:

(a) For streptococci—0.005% and 0.01 sodium azide were added to broth Ia.
(b) For staphylococci—100 g. of sodium chloride were added to 1,000 ml. of broth Ia *(9)*.
(c) For salmonellae—Selenite broth.

Composition of stuffing. A moist and a dry stuffing differing in the amounts of shortening, eggs, and turkey broth were used. The constituents were as follows:

Ingredient	Unit	Type of stuffing	
		Moist	Dry
Bread, dry ½" cubes	Quart	6	6
Giblets, chopped, cooked	Pint	1	1
Celery, chopped	Pint	1	1
Onion, chopped	Cup	1	1
Salt	Tablespoon	1	1
Pepper	Teaspoon	1	1
Poultry seasoning	Teaspoon	4	4
Shortening, melted	Cup	0.66	1.25
Eggs, beaten	.....	4	5
Turkey broth	Pint	1	0

Inoculation. The desired volumes of 24-hour cultures were added to the turkey broth for inoculation of the moist stuffing and to the eggs for the inoculation of the dry stuffing. The inoculated liquids were then sprinkled on the stuffing which was thoroughly mixed with each addition of aliquots of the inoculum. Plate counts of this inoculated, unroasted stuffing revealed that a satisfactory dispersion of the organisms was achieved. However, in experiment 5, Table 1, and in another untabulated experiment the streptococcus plate count of the dry stuffing was roughly 10% of that expected. In several experiments subsequently conducted with different batches of eggs to investigate this particular observation, the results, although variable, indicated that the dispensing egg medium could frequently be highly bactericidal for this streptococcus. The bactericidal activity resided exclusively in the egg white. The exact age of these eggs was not known, but it was felt that the bactericidal activity might depend upon age. Thereafter eggs for dry stuffing experiments were stored in the refrigerator for at least 2 weeks before use, and no further difficulties were noted.

Analytical procedures. Duplicate 11-g. samples of the uninoculated stuffing, triplicate samples of the inoculated, unroasted stuffing and duplicate samples of the central, interior portions of the roasted stuffing in the body cavity were macerated by Waring blendor in 99 ml. of water and plated on the control and selective agars. Duplicate or triplicate tubes of each of the selective broths were inoculated with about 1-g. samples of the roasted stuffing. These latter samples were taken before the respective plating sample. After 24 hours' incubation, the broths were streaked on the appropriate agar media. In addition one sample of roasted stuffing was incubated for 48 hours at 86°F. (30°C.) and another at 50°F.(10°C.) for at least 10 days, macerated and streaked on the agar media. Except as otherwise specified, all incubation temperatures were 86°F. (30°C.) and plates were discarded as negative only after 7 days' incubation. In those experiments where no growth occurred in the selective media, colonies on the total

TABLE 1

Temperature required to destroy streptococci, staphylococci and salmonellae in the center of stuffed turkey

Experiment No.	Ready-to-cook wt. of bird	Wt. of stuffing	Type of stuffing	Initial °F. temp.		Roasting time[a]	Initial plate count per g. of inoculated stuffing[b]	Survival per g. of roasted stuffing[c]	Highest °F. temp. of center of stuffing[d]
				Stuffing	Thigh				
	lb.	*lb.*							
1	11⅝	3 9/16	Moist	78	70	3(30)	380,000 streptococci 700,000 staphylococci 740,000 salmonellae	0 0 0	162
2	11¼	3	Moist	51	58	3(15)	Stuffing inoculated with 160,000 streptococci and incubated separately at 50°F.(10°C.) for 22 hrs. Final count was 440,000.	0	161
3	11 7/16	4 7/16	Moist	61	64	3(20)	640,000 streptococci 2,513,000 staphylococci 866,000 salmonellae	30,000 + 0	152
4	10	3½	Moist	37	37	4 1/6(15)	20,000 streptococci 21,000 staphylococci 51,000 salmonellae	0 0 0	162
5	11⅞	3⅞	Dry	64	62	3(15)	100,000 streptococci 830,000 staphylococci 600,000 salmonellae	+ 0 0	154
6	11⅞	4	Dry	61	55	3¼(15)	2,750,000 streptococci 930,000 staphylococci 970,000 salmonellae	6,900 0 0	157
7	10¼	2¾	Dry	53	56	3 1/6(17)	890,000 streptococci	0	168
8	13 13/16	4⅝	Moist	<32	<32	7(15)	Not inoculated	....	167
9	21½	8 3/16	Moist	54	55	5¼(15)	Not inoculated	....	173
10	23⅛	4 5/16	Dry	....	....	5 5/6(10)	Not inoculated	....	161

[a] Figure outside the parentheses represents hours of roasting in 300°F. oven. Figure inside parentheses represents minutes bird remained at room temperature after roasting and before slicing open for analysis.

[b] *Staphylococcus aureus* 210 used in experiments 1, 5; 196 used in experiments 3, 4, 6, 7.

[c] + indicates survival of less than 10 organisms per g.

[d] This maximum temperature was always achieved during the interval the bird remained at room temperature after the roasting process.

count agar were examined by the Gram stain. In no instance did this procedure reveal survival of the inocula when the selective media failed to do so. Streptococci that survived the roasting process were identified on the basis of the Gram stain, negative catalase test, and growth at 10°C. Staphylococci were identified on the basis of the Gram stain, positive catalase test, and chromogenesis. Only in experiment 3, Table 1, did growth occur in the selective media used for salmonellae. Subsequent reaction in Kligler's Iron Agar revealed that these organisms were not salmonellae.

Roasting procedure. Frozen, ready-to-cook birds of relatively uniform quality were purchased from several supply houses with no particular selection of sex or breed except that the birds were not of the broad-breasted variety. The birds were thawed at room temperature for about 8 hours, refrigerated overnight, and used the following day. The stuffed birds were placed on wire racks in an oval speckled enamel roasting pan and roasted at 300°F.(149°C.) in a preheated Roper gas oven range model 8810 K. Iron Constanton thermocouples were used with a Brown portable potentiometer model 1117. Duplicate thermocouples were inserted into the center of the stuffing in the body cavity. In several experiments a 12-inch Lanco Fahrenheit thermometer in a protective metal shield (Cenco No. 19298) was also inserted into the stuffing. At the completion of the roasting process, the turkey was removed from the oven, allowed to stand at room temperature as indicated in Table 1 and then cut open for bacteriological analysis.

RESULTS AND DISCUSSION

Table 1 contains the data for 7 inoculated and 3 uninoculated, stuffed, roasted turkeys. It can be seen that for both the moist and the dry stuffing a minimum temperature of 160°F.(71°C.) must be reached in the center of the stuffing before the bird is removed from the oven to assure adequate destruction of the organisms tested. As evidenced in experiments 3, 5, and 6, the streptococci are the most heat tolerant.

Figure 1 gives the stuffing temperature curves observed in several representative experiments. It should be noted that under these experimental conditions the temperature of the center of the stuffing rises 5-10°F.(2.8-5.6°C.) during the period the bird stands at room temperature after the roasting process. The most effective lethal temperatures, then, with respect to the center of the stuffing, are obtained after the bird is removed from the oven. It would appear highly desirable that a minimum of 20 minutes be allowed to elapse between removal of the bird from the oven and serving.

Lowe *(8)* stated that heat penetration is slower in a dry than a moist stuffing. Examination of the curves for experiments 1 and 6 in Figure 1 indicate that the rates of heat penetration are about equal in the 2 types of stuffing. Data for the other experiments not graphed also support this observation. A possible explanation for this is that the dry stuffing used in these experiments was actually quite moist due to the thorough mixing administered in adding the inocula.

The importance of achieving a lethal temperature during the roasting process was demonstrated in experiment 3. In this experiment a sample of the center of the stuffing, withdrawn when the temperature reached 140°F.(60°C.), revealed that more than 500,000 staphylococci and more than 50,000 salmonellae survived per g. of stuffing; and that whereas the initial inoculum of streptococci was 640,000, the count in the withdrawn sample was greater than 4,500,000 per g. There was, then, an appreciable multiplication of streptococci during this period of the roasting process. This group of organisms grows well though slowly at 50°F.(10°C.) and rapidly at 113°F.(45°C.). If one arbitrarily assumes that these strepto-

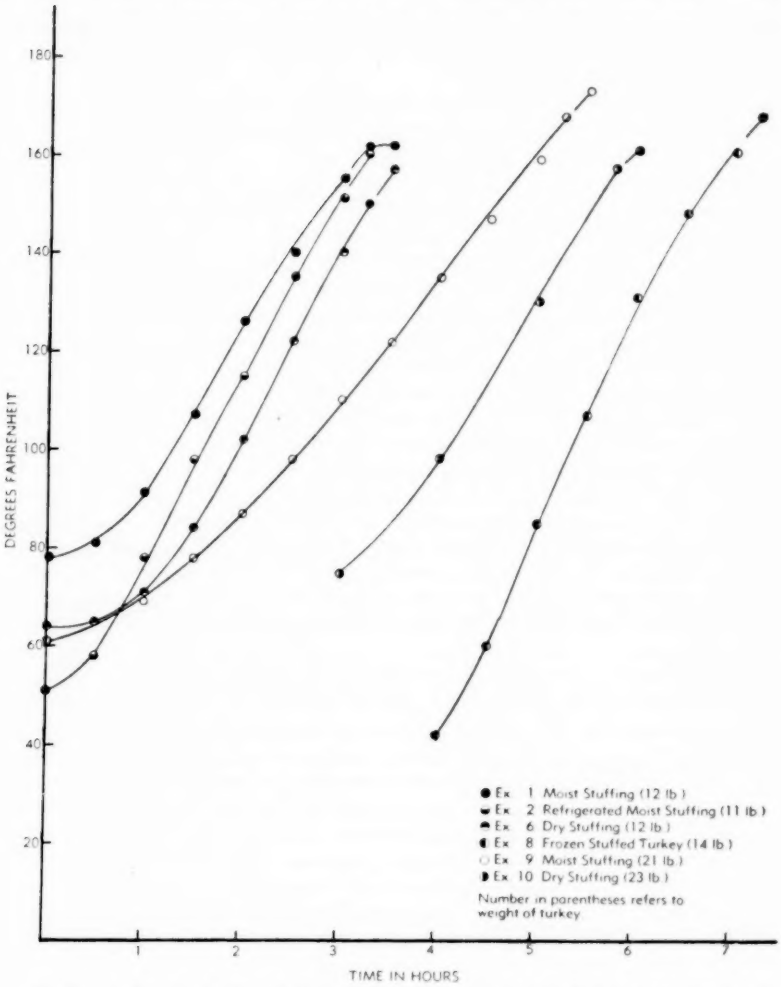

Figure 1. Record of temperature rise in the center of stuffed turkeys roasted at 300°F. The disconnected, uppermost portions of the curves represent the post oven temperature rise observed when the roasted bird is held at room temperature.

cocci actively multiply in a temperature range of 60-120°F.(15-49°C.), it can be observed from Figure 1 that an incubation period of 2½ and 3½ hours was afforded in experiments 6 and 9 respectively. In other words the major part of the roasting process served as a period for microbial growth. These two curves, furthermore, demonstrate that the rate of heat penetration to the center of the stuffing is slower for the larger 21-lb. bird (No. 9) than for the smaller 12-lb. bird (No. 6). In view of the rapid growth of streptococci during the incubation period offered during the

roasting process and the relatively high heat tolerance of this group of organisms, it is imperative that the bird be roasted to the point where a lethal temperature in the stuffing is reached. The practice of partially roasting a turkey at one time and then finishing it off with a slight roasting process at a later date would appear to be particularly unsound from a bacteriological point of view.

The effects of several other factors which may affect the roasting process required to achieve a safe temperature are also indicated in Table 1. A comparison of experiments 2 and 3 indicates that slightly longer roasting periods are required with increased amounts of stuffing. However, most of the present recommendations on amount of stuffing to be incorporated advocate a loosely packed stuffing in order to accommodate the expansion which takes place in the process of roasting and to improve flavor.

Experiments 4 and 8 in Table 1 demonstrate the importance of the initial temperatures of the stuffing and bird in determining the roasting time required to reach a safe temperature in the interior of the stuffing. It will be noted in experiment 4 in which both the stuffing and the bird started at 37°F.(2.8°C.) that, although this was the smallest bird in its weight class, an hour more of roasting time was required for the stuffing to reach the temperature established for the larger birds in experiments 1 and 2. The effect of low initial starting temperatures on prolonging the roasting process required for the stuffing to reach a lethal temperature is, of course, quite pronounced in the case of frozen, stuffed birds. It will be observed in experiment 8 that the roasting time required is about doubled.

In another experiment not recorded in the table, an 11⅛-lb. bird with 2¼ lb. of dry stuffing, both at initial temperatures of —4°F.(—20°C.), was roasted at 325°F.(163°C.). A period of 5¾ hours was required for the temperature of the center of the stuffing to reach 165°F.(74°C.). A comparison of the temperatures of the thigh and the stuffing revealed that when the thigh temperature reached 200°F.(93°C.) after 3½ hours of roasting, the temperature of the stuffing was only 98°F.(37°C.). This indicates that the use of the present recommended thigh temperature of 190°F.(88°C.) as a criterion of sufficient roasting does not correlate with a safe temperature in the stuffing where birds are roasted directly from the frozen, stuffed state.

It was of further interest to observe with this identical stuffed bird that in the process of freezing prior to roasting, 3¾ hours were required to lower the temperature of the stuffing from 66°F.(19°C.) to 45°F.(7.2°C.) in a freezer operating at —8°F.(—22°C.). The initial thigh temperature in this case was 74°F.(23°C.). With the bird in experiment 4, 6½ hours were required for the temperature of the stuffing to drop from 64°F. (18°C.) to 45°F.(7.2°C.) in a refrigerator operating at an average temperature of 38°F.(3.3°C.) during that period. The initial thigh temperature was 60°F.(14°C.). It can readily be appreciated that the prompt refrigeration of stuffed turkey is not easily achieved.

A comparison of roasting processes currently advocated revealed that considerable variation exists. For example De Gouy *(4)* recommended roasting at 450°F.(232°C.) for 15-20 minutes, reducing the temperature

to 350°F.(177°C.) and roasting for a period based on 20-25 minutes per lb. The U. S. Department of Agriculture *(13)* recommended roasting birds of 9-12 lb. ready-to-cook weight at 325°F.(163°C.) for 3-3½ hours and those of 21-26 lb. weight at 300°F.(149°C.) for 6-7½ hours. Bradley has recommended roasting at 450°F.(232°C.) for 30 minutes, reducing the temperature to 350°F.(177°C.) and roasting for a period based on 18 minutes per lb. Simpson and Taylor *(11)* recommended that turkeys of 10-14 lb. be roasted at 325°F.(163°C.) for 18-20 minutes per lb. and that birds heavier than 18 lb. be roasted at 300°F.(149°C.) for 13-15 minutes per lb. The Poultry and Egg National Board *(10)* recommended birds of ready-to-cook weight of 8-12 lb. be roasted at 325°F.(163°C.) for 4-4½ hours. Those of 20-24 lb. weight should be roasted at 300°F.(149°C.) for 5½-6 hours. Swift and Company *(12)* recommend 4-4½ hours for 11- to 13-lb. birds and 7½-8½ hours for 20- to 24-lb. birds at 325°F.(163°C.).

It will be noted that the roasting processes recommended by U.S.D.A., Simpson and Taylor, and the Poultry and Egg National Board are less than the others. A comparison of these 3 recommended roasting processes with that used in the present experiments reveals that at least with the smaller birds they should generally result in a safe temperature in the stuffing. A possible exception arises in the case of birds with low initial temperatures as in experiment 4. The recommended roasting process for this bird may be interpreted as being 3, 3½, and 4 hours at 325°F.(163°C.) whereas experimentally the time required was 4⅙ hours at 300°F.(149°C.). In the case of the two larger birds in experiments 9 and 10 these recommended roasting procedures are just barely adequate to assure a safe stuffing temperature. Considering the recommendations for the bird in experiment 9, roughly 6, 5⅓, and 5½ hours' roasting at 300°F.(149°C.) are advocated. The experimental roasting time was 5¼ hours at this same temperature. It is readily conceivable that had this bird started at lower initial temperatures the recommended roasting processes would not then be sufficient to arrive at a safe temperature in the stuffing.

Some recommendations stipulate a thigh temperature of 190°F.(87.8°C.) as a criterion of satisfactory roasting. However, Alexander *et al. (1)* claimed that their experimental observations were not, in their opinion, sufficiently uniform to warrant the recommendation of a specific thigh temperature. Lowe *(8)* stated that for small chickens stuffing temperature is preferred to the thigh or breast temperatures. Unfortunately, the conventional household meat thermometer may be too short to accurately determine the temperature of the center of the stuffing for a large bird. Nevertheless, it is felt that the use of accurate stuffing temperature is a safer criterion and is better qualified to reflect the effects of different variables.

SUMMARY

The temperature required to kill streptococci (enterococci), staphylococci, and salmonellae experimentally added to turkey stuffing and several factors affecting the attainment of this temperature have been studied, resulting in the following observations:

A temperature of 165°F.(73.9°C.) reached in the center of the stuffing during the roasting period appears sufficient to kill the above organisms and seems to allow a modest margin of safety.

A post oven temperature rise of 5-10°F.(2.8-5.6°C.) occurs in the center of the stuffing if the roasted bird is allowed to remain at room temperature for 20 minutes. A practice of allowing this temperature rise to take place would appear to be desirable.

An active bacterial multiplication occurs during the earlier phases of the roasting process. This period of multiplication is longer in the larger birds where the rate of heat penetration into the stuffing is slower. The roasting process should, therefore, not be interrupted until the lethal temperature (165°F.) is reached in the stuffing.

Increased amounts of stuffing tend to increase slightly the time required to reach the lethal temperature.

The initial temperatures of the stuffing and the bird exert an appreciable effect on the time required to reach the lethal temperature. The lower the initial temperatures, the longer the roasting period required. In the case of frozen, stuffed birds the time required may be more than twice that required for unfrozen stuffed birds.

Present recommended roasting processes appear to be generally adequate for smaller birds. For birds over 18 lb. weight, there is very little, if any, margin of safety in some recommendations.

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THE FREE AMINO ACIDS OF MUSTS AND WINES. I. MICROBIOLOGICAL ESTIMATION OF FOURTEEN AMINO ACIDS IN CALIFORNIA GRAPE MUSTS

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Present knowledge indicates that amino acids may be of basic importance in the production, flavor, and quality of fermented beverages. However, the literature contains no information on the amounts of specific amino acids in grapes and wines. A limited amount of quantitative data exists on amino nitrogen in musts and wines, and several amino acids have been identified in musts. The literature prior to 1951 on the nitrogen content of musts and wines has been summarized by Amerine and Joslyn *(1)*. Since then Valaize and Dupont *(21)* reported on the amino nitrogen content of principal grape varieties in France, and identified 7 amino acids by paper chromatography. More recently Castor and Guymon *(5)* reported the amounts of free isoleucine, leucine, and valine in a grape must, in connection with studies on the formation of higher alcohols during fermentation.

The present paper reports quantitative estimation of the amounts of 14 free amino acids found in the must of 7 varieties of grape produced in California. The *Lactobacillus* method of microbiological assay was employed, partly because of the importance of these organisms in wine spoilage, as indicated by Cruess *(6)*, and partly because of the availability and convenience of the method. A problem encountered in applying this method directly to must samples and the results of attempted solutions are reported.

EXPERIMENTAL PROCEDURE

Varieties of grapes assayed. Liquid musts (juice pressed from the crushed grapes) of the varieties of *Vitis vinifera* shown in Table 1 were assayed.

TABLE 1

Grape variety	Skin color	Must color	Grape type
Aglianico	Red	White	Wine
Cabernet Sauvignon	Red	White	Wine
Tannat	Red	White	Wine
Flame Tokay	White	White	Table[a]
French Colombard	White	White	Wine
Sauvignon blanc	White	White	Wine
Thompson Seedless	White	White	Table[b]

[a] A table grape which is also used for wine production in California.
[b] A raisin grape which is also used as table fruit and for wine production in California.

Preparation of samples. About 5 pounds of each of the varieties of grape, except French Colombard, were stripped from freshly harvested clusters of ripe fruit. Intact, undamaged fruit was selected and stems were discarded. The grapes were crushed and pressed by hand and the skins and seeds discarded. The must was stored under toluene

at about 0°C. until assays were completed. The French Colombard sample was obtained from a 60-gallon lot of fresh must.

The low pH (3.4 to 3.7), content of organic acids (chiefly tartaric and malic) and the wide variation in content of some amino acids of the musts required additional preparation of samples for assay. The must samples were brought to room temperature and shaken to suspend or redissolve any substances precipitated in cold storage. A portion of each sample was adjusted to pH 7.0 with sodium hydroxide solutions. Dilution of the sample did not exceed 3 cc. of alkali per 500 cc. of sample. Further dilution of the samples was required to bring their amino acid activity, estimated by preliminary trial, within the range of certain assays. Samples were diluted to 1:100 to 1:10 for arginine, and 1:10 for glutamic acid, histidine, isoleucine, leucine, phenylalanine, tryptophane, tyrosine, and valine. Dilutions were made in standard volumetric glassware at 20°C., and samples were rechecked for pH value before use in assays.

TABLE 2

Amino acid assay methods used

Assay and standard used	Method reference
L-Arginine HCl	McMahon *et al. (16)*
L(+)-Aspartic acid	Stokes *et al. (20)*
L-Cystine	Dunn *et al. (7)*
L(−)-Glutamic acid	Lyman *et al. (15)*
Glycine	Shankman *et al. (18)*
L(−)-Histidine HCl, monohydrate	Dunn *et al. (9)*
DL-Isoleucine	Kuiken *et al. (14)*
L(−)-Leucine	Kuiken *et al. (14)*
DL-Lysine HCl	Dunn *et al. (7)*
DL-Methionine	Dunn *et al. (11)*
DL-Phenylalanine	Dunn *et al. (10)*
DL-Tryptophane	Dunn *et al. (8)*
L-Tyrosine	Shankman *et al. (17)*
DL-Valine	Kuiken *et al. (14)*

Assay methods. The methods used for assaying the individual amino acids are cited in Table 2. Commercially available dry, premixed basal media were employed. Results of isoleucine, leucine, and valine assays, using these basal media, compared favorably with results obtained with basal media prepared from basic components in this laboratory. Samples were assayed in duplicate at 3 levels, 0.5, 1.0, and 1.5 ml. per assay tube, except for French Colombard samples which were assayed at 2 levels. Total amount of basal medium, standard or sample and distilled water in each tube was 5 ml. The contents of each tube were thoroughly mixed before autoclaving, by means of a vibrotool. The tubes were capped with glass vials, according to Stier and MacIntyre *(19)*, to avoid contamination of the assays with substances from cotton stoppers. Inoculum was cultured in centrifuge tubes containing 5 ml. of basal medium supplemented with the amino acid to be assayed. After incubation the inoculum was washed 3 times in the centrifuge tube with 0.9% saline and diluted to barely visible turbidity with saline. One drop of the inoculum suspension was delivered to each standard and assay tube from a hypodermic syringe fitted with a 22-gauge needle. Assays were incubated as required by the particular method used (Table 2). Aseptic technique was observed throughout the procedures. Assays were completed by electrometric titration of the acid produced by assay organisms, because of the color and turbidity of the added samples.

Special preliminary treatment of samples. In a number of assays definite upward or downward "drift" of the final calculated assay values was noted. The occurrence and nature of the drift is shown in Tables 3 and 4. The drift appeared to be related to the amount of sample added to the tube, indicating the presence of factors in the sample which could interfere with or possibly stimulate multiplication or lactic acid production of the bacteria.

An attempt was made to eliminate the interfering factors by means of simple preliminary treatment of the samples. The usual chemical clarification methods were not used, in order to prevent possible occlusion of amino acids in voluminous precipitates or to avoid introduction of substances which could further interfere with the assays. Two treatments were tried. The first consisted of gently boiling the neutralized sample, filtering, washing the filter, and adjusting the filtrate and washings to volume with distilled water. The heat treatment was based on the finding, made during the course of this work, that grape seed tannin could depress acid production in assay standard tubes. Heat converts certain tannins to insoluble substances which could be removed by filtration. Samples were neutralized before boiling to avoid hydrolysis of albuminoids and peptides, reported in wines by Hennig *(12)*.

TABLE 3

The nature and effect of "drift" on must assays, shown as final calculated values for amino acids

(mg. per 100 ml. of must)

Assay tube No.	Amount of sample (ml.)	No effect	Downward drift			Upward drift
		(Leucine)	(Glutamic acid)	(Aspartic acid)	(Cystine)	(Isoleucine)
1	0.5	5.2	58.0	4.1	0.48	4.2
2	0.5	5.2	52.0	4.1	0.42	4.0
3	1.0	5.6	49.0	3.3	0.30	4.7
4	1.0	5.6	46.5	3.2	0.34	4.5
5	1.5	5.5	34.0	2.8	0.11	4.9
6	1.5	5.3	35.0	2.3	0.13	4.7

The second treatment consisted of diluting the neutralized sample with 5 volumes of 95% alcohol, in accordance with the method used by Joslyn and Stepka *(13)* to extract amino acids from fruit and decrease the amount of contaminating substances. The mixture was allowed to stand several hours at room temperature and filtered. To remove alcohol it was reduced to a small volume *in vacuo* at 50° to 60°C., and adjusted to the volume of original sample with distilled water.

To further investigate the cause of the drift in final assay values, assays of a hydrolyzed casein solution were run parallel with must assays for 9 amino acids. The effect of grape seed tannin on the standard curves of several assay methods was studied. Tannin was added to the basal medium in concentrations to give 0.1, 1.0, 2.0, and 6 mg. per tube. For each of 7 amino acids, (4 different assay organisms) a series of standard tubes was set up at each level of tannin concentration. The tannin standard series were inoculated and completed in the same manner as standard series for must assays.

RESULTS AND DISCUSSION

Free amino acids in musts. Table 4 shows the amounts of free amino acid activity found in the musts of the 7 grape varieties examined. The values in the table are averages of duplicate samples assayed at 3 levels. Glutamic acid and arginine were on the average present in amounts considerably greater than those of the other 12 amino acids. Arginine, tyrosine, and glutamic acid showed the greatest variation in content among the 7 varieties of grape, while methionine, lysine, and leucine showed the least. Comparison of the variation in amino acid content of the different grapes with the number of cases of drift for each amino acid did not indicate any apparent relationship between variation and drift. Drift occurred more frequently in the methionine assay, where the variation in amino acid content was smallest among the 7 varieties, than in the arginine assay where the variation was greatest.

Of the 9 hydrolyzed casein assays run parallel with must assays, 5 showed no appreciable drift; 3 showed a slight upward drift, and one a considerable downward drift. Assays for glycine, methionine, and valine in hydrolyzed casein showed a slight upward drift, which is correlated with drift in must assays only in the case of valine. Only the assay for histidine in casein exhibited considerable downward drift, while must assays for histidine showed only one case of downward drift in the 5 varieties of grape assayed. Since significantly large amounts of downward drift occurred infrequently in hydrolyzed casein assays, but rather frequently in must assays, the idea that the must samples contained some factor or factors which tended to inhibit acid production by the assay organisms appeared to be confirmed.

TABLE 4

Free amino acid activity, found by microbiological assay, in the must of seven varieties of grape

(mg. per 100 ml. of must)

Amino acid	Aglianico	Cabernet Sauv.	Flame Tokay	French Colombard	Sauvignon blanc	Tannat	Thompson Seedless	Average activity
Glutamic acid	68.0[c]	33.5[c]	106.7[c]	26.5	57.3[c]	88.9[c]	45.8[c]	68.7
Arginine	113.0[d]	8.8[c]	15.2	103.0	14.8[c]	7.0	20.1	40.3
Histidine	9.4	5.7[c]	....	8.3	14.3	8.5	....	9.2
Isoleucine	3.6[c]	4.5[c]	3.1	7.5	10.0	3.4	3.8	6.6
Leucine	4.6[c]	6.8	5.4	7.0	7.7[c]	4.2	8.1	6.2
Valine	3.6[c]	6.1	4.2	9.4	8.8	4.1	5.2	6.0
Aspartic acid	5.4[c]	2.2	9.9	6.0	3.8[c]	5.7[c]	3.3[c]	5.2
Phenylalanine	6.6[d]	2.9	3.1	4.0	7.1[c]	7.8[c]	4.5[c]	5.1
Tryptophane	5.6[c]	2.2[c]	3.7	3.1	6.4[c]	6.5[c]	5.6[c]	4.7
Tyrosine	2.9[c]	2.2	1.1[c]	4.6	0.7[c]	1.5[c]	1.0	2.0
Lysine	1.4[c]	1.9[c]	2.0[c]	1.1	1.6[c]	1.7[c]	1.8	1.6
Methionine	1.4[c]	1.6[c]	1.1	1.5	1.6[c]	1.8[c]	1.0[c]	1.4
Glycine	0.4[c]	0.6	0.8	1.4	0.5[c]	0.3	0.6	0.7
Cystine	0.3[c]	0.4[c]	0.5[c]	0.4	0.3	0.2	0.4	0.4

[c] Progressive downward drift apparently related to level of sample.
[d] Highest value found after preliminary treatment of sample with heat or alcohol.
[e] Slight progressive upward drift.

Effect of special preliminary treatment of samples. Table 5 shows the results of preliminary heat or alcohol treatment of samples. Boiling the sample produced a significantly large increase in assay value with arginine alone. A boiling time of 2 minutes yielded the best results. Increasing the boiling time for 2-minute steps up to 8 minutes often gave decreased values. Recovery experiments were run with arginine and glutamic acid added to the must. The results indicated that in Aglianico must, heated for 2 minutes, the final calculated assay values represented about 80% of the arginine present. (In treated and untreated French Colombard must, the final assay values represented 90% of the glutamic acid present.) The results of the methionine assay were not affected by boiling. Results of the 6 other amino acid assays indicated that, although downward drift appeared to be eliminated, the preliminary heating of the sample reduced the final assay value below that for the untreated sample.

With the exception of arginine, isoleucine, phenylalanine, and methionine, the treatment of must samples with alcohol gave assay results gen-

erally less than that of the untreated samples. Downward drift appeared to be eliminated, except for arginine, but a slight upward drift appeared in the case of the methionine assay.

TABLE 5

Effect of heat and alcohol treatments on assay results with Aglianico must

Amino acid	(mg. amino acid activity per 100 ml. of must)		
	Treatment of sample		
	None	Boiled	Alcohol
Arginine	4.9[f]	113.0	104.5[f]
Histidine	9.4	7.8	7.6
Isoleucine	3.6[g]	....	3.6[g]
Leucine	4.6[g]	....	3.1[g]
Valine	3.6	3.3	2.2
Aspartic acid	5.4[f]	4.8	4.7
Phenylalanine	4.1[f]	2.9	6.6
Tryptophane	5.6[f]	5.4	5.1
Tyrosine	2.9[g]	1.8[g]	2.5[g]
Methionine	1.4[f]	1.4[f]	1.5[g]

[f] Progressive downward drift.
[g] Progressive upward drift.

Tannin as an inhibiting factor. The general effect of the tannin on the standard curves is shown in Table 6, as per cent depression of the curve. The data of Table 6 also include the range of concentrations of tannin probably present in must assay tubes. These values were calculated from extensive work by Amerine and Winkler *(2)* on the tannin content of wines from these and other grapes. As the results of work by Berg *(3)* indicate that there is little change in tannin content during fermentation of musts to wines, it is likely that the calculated ranges of concentration are correct. It appears from the results of Table 6, that grape seed tannin, in the amounts present in some of the must samples, could strongly depress the acid production of several assay organisms. However, comparison of Tables 4, 5, and 6 indicates that there is little correlation between the ability of grape seed tannin to decrease acid production in specific inhibitor experiments, and the downward drift found in assays of must samples. In the case of arginine, where the greatest inhibition was found in Aglianico must (Table 5), the tannin content of the must, because of the dilution of the sample, was well below that which gave an 8% depression of the standard curve (Table 6).

In the case of valine, in Aglianico must, an upward rather than a downward drift was found in the final calculated amino acid activity value, while Table 6 indicates a better than 10% average depression of the standard curve with the amount of tannin which could be expected per tube. In the methionine assay in Aglianico must downward drift occurs, but its effect on the final assay value is not as severe as would be expected from the data of Table 6. For these reasons it was considered that the effect of tannin on the assay organism may play some part in producing the downward drift of assay values, but it is not clear that it is the primary factor involved. The nature of other factors which might cause the drift of assay

values is not understood. Unreported work by Castor *(4)* indicated that other components of wines, such as tartaric and malic acids, glycerol, and alcohol, in amounts as large as those found in musts and wines, did not affect the standard curve of microbiological assays for riboflavin and pantothenic acid.

SUMMARY AND CONCLUSIONS

The amounts of the amino acids, arginine, aspartic acid, cystine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, tryptophane, tyrosine, and valine, were estimated by microbiological assay in the must of 7 varieties of grapes. Glutamic acid and arginine are on the average the amino acids present in largest amount.

TABLE 6

Per cent depression of amino acid assay standard curve by grape seed tannin

Amino acid	Assay organism	Added grape seed tannin per assay standard tube (mg.)				Range of tannin concentration in must assay tubes (mg.)[b]
		0.1	1.0	2.0	6.0	
		% depression std. curve				
Arginine	*L. casei* e	8	73	100	....	0.03-0.002
Tyrosine	*L. casei* e	27	69	100	....	0.3 -0.02
Histidine	*L. fermenti* 36	13	50	100	....	0.3 -0.02
Methionine	*L. fermenti* 36	....	....	32	100	3.0 -0.2
Leucine	*L. arabinosus* 17-5	....	....	100	....	0.3 -0.02
Valine	*L. arabinosus* 17-5	11	62	89	100	0.3 -0.02
Aspartic acid	*Leuc. mesenteroides* P-60	....	....	14	36	0.3 -0.02

[b] Calculated values; see text.

Considerable downward drift in final calculated assay values was found in some assays, and a relatively slight upward drift in others. Several parallel assays of hydrolyzed casein indicated that the cause of the drift might be substances in the must samples. Preliminary treatment of samples by boiling, or dilution with ethanol, to inactivate or precipitate substances interfering with the assay was notably successful only in the case of arginine and phenylalanine. Grape seed tannin, in the amounts corresponding to the tannin in musts similar to those assayed, was found to interfere with the acid production of the assay organisms. Lack of correlation between specific inhibitor experiments and the results of actual must assays indicate that tannin is probably not the only factor concerned.

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THE FREE AMINO ACIDS OF MUSTS AND WINES. II. THE FATE OF AMINO ACIDS OF MUST DURING ALCOHOLIC FERMENTATION

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The amounts of 14 free amino acids found in 7 varieties of California grapes were reported in Part I of this study *(3)*, but a lack of information in the literature on the fate of the individual amino acids of must during conversion of the must to wine by alcoholic fermentation indicated that data on this subject would be useful also.

Extensive studies by Nielsen *(10)*, Thorne *(16)*, and Barton-Wright and Thorne *(2)* have shown that the total and amino nitrogen and the amino acids of beer wort were largely decreased during the early stages of fermentation of the wort. A decrease of total and organic nitrogen has been noted during wine fermentation. Hennig and Ohske *(8)* found that albuminoid, ammonia, phosphomolybdate precipitable, amino and humin nitrogen decreased, while amide and certain unidentified nitrogen fractions remained unchanged. During the later stages of the fermentation of must the total nitrogen, including ammonia, phosphomolybdate precipitable, and amino nitrogen in the must slowly increased in amount. The decrease of nitrogenous fractions during early fermentation was attributed to consumption by yeast, and the increase in the later stages to its release from the yeast cells, presumably by cellular autolysis. Peynaud *(11)* and Barbera *(1)* also reported decrease of organic nitrogen during must fermentation. The report of Hennig *(7)* appeared to be the best source of information on the amounts of general classes of organic nitrogen residual in wines after fermentation. Hennig reported that phosphomolybdate precipitable, amino and unidentified nitrogen fractions accounted for 31, 30 and 26%, respectively, of the total nitrogen of wine, while albuminoid, ammonia, humin and amide nitrogen together accounted for 13%. Since phosphomolybdate precipitable nitrogen compounds include some amino acids it appears that amino acids account for over 30% of the organic nitrogen of wines. However, no information was found in the literature on the residual amounts of specific amino acids in wines after fermentation.

Increasing knowledge of the importance of amino acids in the nutrition of yeasts and lactic acid bacteria, contributed by Thorne *(16)*, Schultz and Pomper *(15)*, and Dunn *et al. (5)*, makes it obvious that residual amino acids are significant for the keeping quality of wines. Both yeasts and lactic acid bacteria are involved in wine spoilage, as indicated by Scheffer and Mrak *(14)* and Cruess *(4)*. The purpose of this paper is to report the changes in the amounts of free amino acids during fermentation of the must. The relationship between decrease in amino acids and two chief biochemical activities in the course of wine fermentation—namely, yeast multiplication and ethanol production—are shown. The residual amounts of amino acids in the young wines are indicated.

EXPERIMENTAL

Fermentation procedure. Sixty gallons of must pressed from freshly picked and crushed French Colombard grapes was placed in a glass-lined steel tank. The must contained 24% reducing sugar and its initial pH was 3.5. It was inoculated with 1.2 gallons of an actively fermenting pure yeast culture. The yeast culture used was *Saccharomyces cerevisiae* variety *ellipsoideus*, "Montrachet strain," (Enology Culture No. 522), originally obtained from the Station Oenologique de Burgogne, Beaune, France. The starter culture was prepared in French Colombard juice sterilized by intermittent heating. After thorough mixing, the initial yeast cell count in the inoculated tank was 5.7×10^6 cells per ml. After inoculation, fermentation was permitted to proceed with no agitation other than that caused by vigorous evolution of carbon dioxide and the stirring preceding each sampling. The fermentation was carried out in a room held at 21° to 22°C. No attempt was made to control directly the temperature of the fermenting must. The initial temperature of the must was 19.7°C.

Sampling procedure. An initial sample of the must was withdrawn immediately after addition and thorough stirring in of the yeast culture. Subsequent samples were withdrawn at 6- to 12-hour intervals. Immediately before each sampling the must was mechanically stirred in order to thoroughly suspend the yeast. Separate samples were taken for ethanol determination, and for yeast cell counts and amino acid assay. Samples for ethanol were distilled at once. Each sample for yeast cell count and amino acid assay consisted of five 150-cc. batches, taken at different levels within the fermenting tank, and combined in a bottle chilled in an ice bath. Each chilled sample bottle contained 15 ml. of purified toluene. A 25-ml. portion of the mixed chilled sample was set aside for yeast cell count and stored at 0°C. until the counts were completed. The remainder of the sample was centrifuged in the cold at 5°C. to remove the yeast. Centrifuged must samples were stored at 0°C. under toluene until the amino acid assays were completed. Fermentation temperature readings were made at each sampling with an industrial thermometer with 24-inch metal-cased stem.

Analyses. Yeast cells were counted with a Levy-Hausser counting chamber. The usual precautions were taken to avoid errors in counting caused by sedimentation, evaporation, and unequal distribution of cells in the chamber. The method used was based on the observations of Richards *(13)*. Cell number, rather than yeast weight, yeast total nitrogen, or turbidity measurements, was used as an indication of the amount of yeast substance formed because of the inherent turbidity of the must, a fraction of which could be removed with the yeast by centrifuging. Amino acid assays were made by microbiological assay as reported in Part I of this study *(3)*. Alcohol was determined by hydrometer in distillates obtained from the samples. Reducing sugar was determined in the must by the method of Underkofler *et al. (17)*.

RESULTS AND DISCUSSION

Changes in the amounts of individual amino acids which occurred during alcoholic fermentation are shown in Figures 1, 2, and 3. The changes followed patterns which appeared to be related to 2 consecutive and overlapping phases of alcoholic fermentation itself. The 2 phases, as described by Lee *(9)*, consist, first, of the multiplication of the microorganism which provides the enzyme systems needed to carry out the second phase, formation of fermentation products. The overlapping relationship of these 2 phases is shown in Figure 1. The curve for yeast cell multiplication exhibited the usual features of the growth of cultures of unicellular microorganisms, according to Porter *(12)*. In Phase I the curves for decrease of 11 of 14 amino acids assayed, coincide with the curve for yeast cell multiplication, but in all but 2 cases the decrease stopped before multiplication. The cessation of yeast cell multiplication marked the end of Phase I. The yeast cell count at that time was 112.5×10^6 cells per ml. Phase II of the fermentation began with the appearance of appreciable amounts of ethanol. At the end of Phase I, about 5% of ethanol by volume was already present.

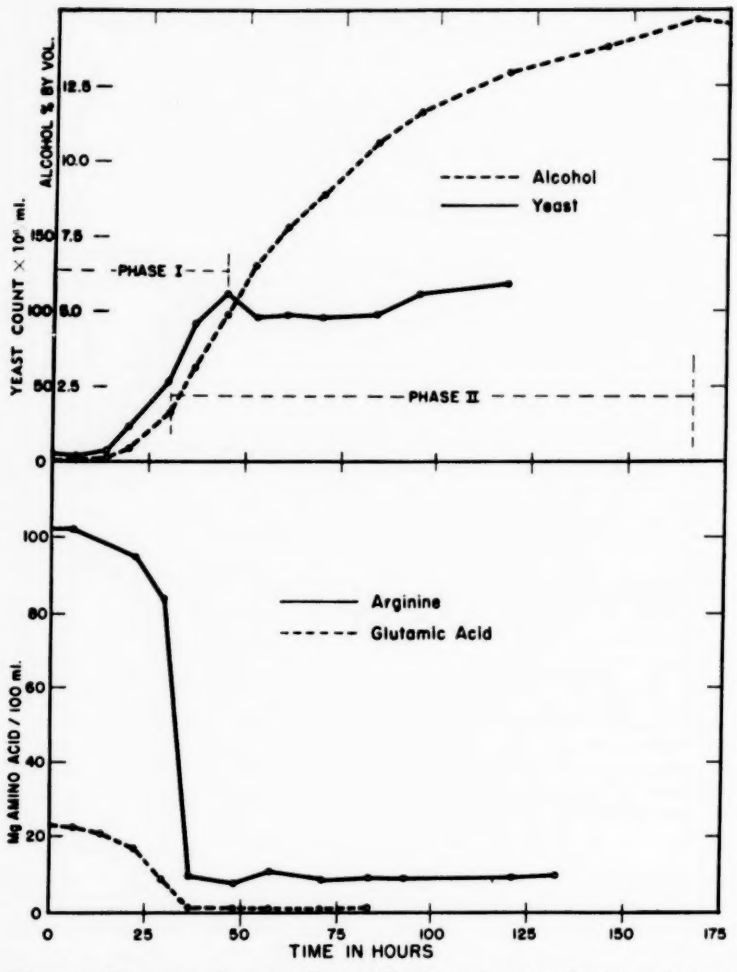

Figure 1. The course of yeast cell multiplication, ethanol production, and decrease in amino acids during fermentation of must to wine.

During the course of the fermentation the temperature rose from 19.5° to a maximum of 29.5°C., reached between the 60th and 70th hours after inoculation, then fell slowly to 19.5°C. as fermentation was completed. All the amino acids except glycine, lysine, and cystine showed a large decrease in amount during Phase I of the fermentation. For 11 individual amino acids the decrease varied between 70 and 95% of the amount initially present in the must. The rapid decrease of arginine, glutamic acid, valine, isoleucine, leucine, aspartic acid, tryptophane, phenylalanine, and methionine was terminated from 10 to 25 hours before yeast cell multiplication

ceased. The decrease of histidine and tyrosine appeared to terminate simultaneously with the cessation of cell multiplication. Glycine, lysine, and cystine showed little or no decrease in amount during Phase I. The fate of arginine was of particular interest because decrease in its amount ceased 12 hours before yeast cell multiplication, and appreciable amounts (10 mg. per 100 ml. of must) remained throughout the fermentation, although Thorne *(16)* has ranked it as a good yeast nutrient.

Changes in the amounts of amino acids during Phase II of the fermentation, after yeast cell multiplication ceased, were much smaller in magnitude than in Phase I. The amounts of arginine, glutamic acid, histidine, methionine, phenylalanine, glycine, lysine, and cystine remained almost constant throughout Phase II. Valine, isoleucine, and leucine definitely increased in amount, and tryptophane and tyrosine increased slightly, in the fermenting must. Although the yeast cell count remained relatively

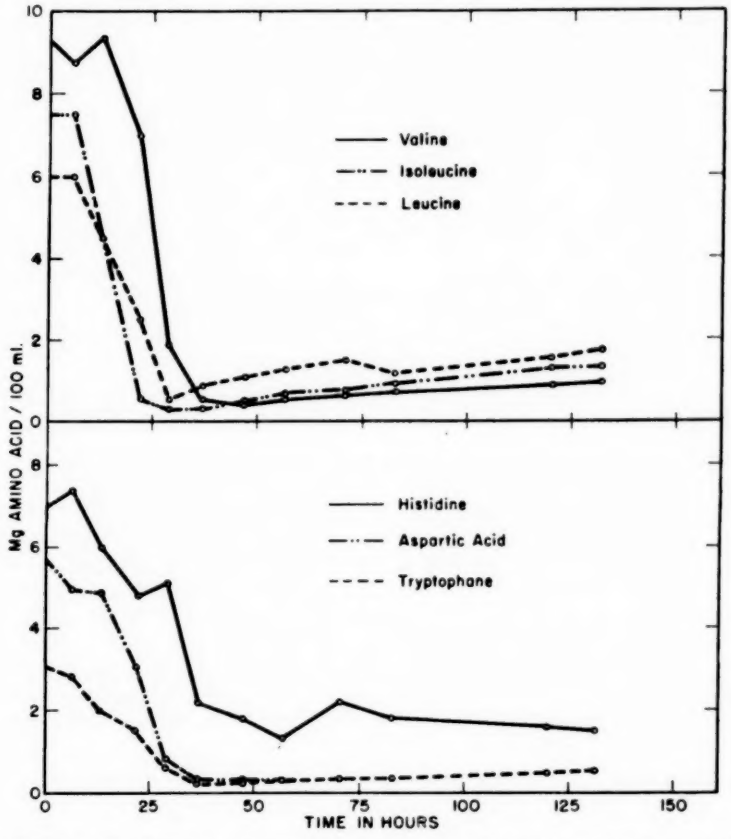

Figure 2. The course of changes in the amount of amino acids during fermentation of must to wine.

static in Phase II, enzyme activity was vigorous as shown by the continued production of ethanol until fermentable sugar had completely disappeared. About 60% of the ethanol produced during the fermentation was formed after the rapid yeast cell multiplication of Phase I had ceased. Under conditions where yeast cell multiplication is prevented but enzyme activity is possible, cellular autolysis presumably occurs. Autolysis could account for the return of several amino acids to the fermenting must, according to Harden *(6)*. The data indicated that 85 to 90% of the group of amino acids considered were removed from the must by the yeast during the fermentation. The small amounts of amino acids returned to the must

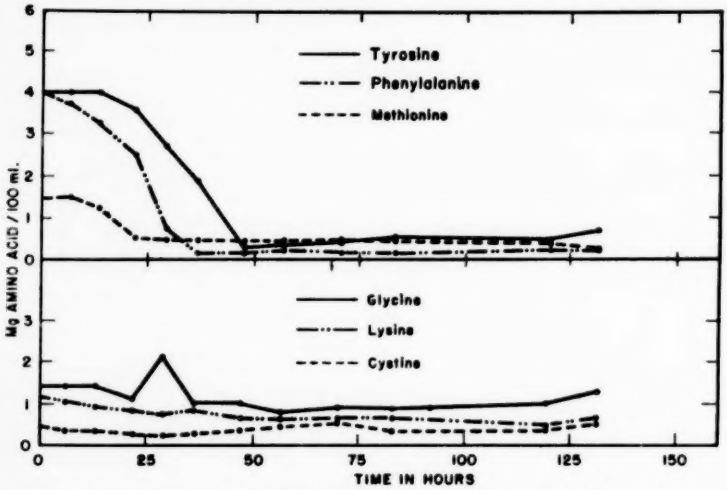

Figure 3. The course of changes in the amount of amino acids during fermentation of must to wine.

during the fermentation were considered in calculating this estimate. It appears, also, that the residual amino acid composition of the wine differs from the amino acid composition of the must, in the proportion of the individual amino acids present, because of the different amounts of each amino acid removed, and the fact that three remained relatively unchanged.

The small amounts of individual amino acids residual in the young wine indicate that it would not support extensive growth of lactic acid bacteria. To obtain extensive growth and acid production in a synthetic, assay-type medium Dunn *et al. (5)* employed 66.7 mg. per 100 ml. of 21 amino acids. The amounts of individual amino acids residual in the wine obtained in the present experiment ranged from 0.8 to 15% of the amounts employed in the assay medium.

SUMMARY AND CONCLUSIONS

The fate of 14 amino acids found in fresh grape must was followed during fermentation of the must to wine. Eleven of the amino acids, arginine, glutamic acid, valine, isoleucine, leucine, histidine, aspartic acid, trypto-

phane, tyrosine, phenylalanine, and methionine, were largely removed from the must by the yeast. Removal of individual amino acids varied from 75 to 90% of the amounts initially found in the must. Three amino acids, glycine, lysine, and cystine, present in the must in small amounts, showed little or no change in amount during fermentation. Five amino acids, valine, isoleucine, leucine, tryptophane, and tyrosine, were returned to the fermenting must in small amounts after yeast cell multiplication had ceased, presumably by yeast cell autolysis. The variations in removal of amino acids indicated that the resulting wine had an amino acid composition differing considerably from that of the must in proportions of individual amino acids present.

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THE THERMAL DESTRUCTION OF PECTIC ENZYMES IN GRAPEFRUIT JUICE[a,b]

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Factors influencing the occurrence of gelation and clarification of frozen citrus concentrates and means of preventing these defects have been studied by a number of investigators *(4, 5, 16, 33, 34, 37, 38)*. Rouse *(37)* reported that gelation was caused by pectinesterase (PE) demethylating pectin to form low-methoxyl pectin which, in the presence of a sufficient concentration of polyvalent cations, reacted to form a pectinate gel. Wenzel, Moore, Rouse, and Atkins *(40)* pointed out that the pectin content, PE activity, and the concentration of polyvalent cations in citrus concentrates were major factors associated with gel formation. They demonstrated that gelation and clarification could be prevented by storing the product at or below —18°C.(0°F.) or by thermal inactivation of PE in the unconcentrated juice. Moore, Huggart, and Hill *(33)* showed that unpasteurized, concentrated grapefruit and orange juice stored for 6 months at —7°, 0°, and 4.4°C.(20°, 32°, and 40°F.) gelled or clarified.

According to Phaff and Joslyn *(35)* the action of PE was first described by Frémy in 1840 who observed that when juice from young carrots was mixed with pectin solution, gel formation invariably took place. Baker *(6)* and Kertesz *(26)* have summarized the results of several investigators who have studied enzyme deesterification of pectin, factors influencing the characteristics of low methoxyl-metallic pectinate gels, and the uses to which low methoxyl gels may be put.

Cruess *(15)* was among the first to report gelation and clarification of citrus juices. While working with orange juice he observed a sample that gelled, later the gel coagulated and settled out carrying the suspended particles of fruit with it, leaving the juice clear. He also demonstrated that pasteurization would prevent settling out of the suspended particles. Joslyn and Marsh *(20)* and Joslyn and Sedky *(22)* demonstrated that pectic enzymes were responsible for clarification. Loeffler *(27)* reported that PE enzymic changes occur rapidly in orange juice. Kertesz *(24, 25, 26)* has studied the clarifying action of tomato PE thoroughly.

Clarification of fruit juices may also occur as the result of depolymerizing enzymes. McColloch and Kertesz *(31)* found an enzyme in tomatoes, which they called tomato pectic acid depolymerase (PD), which under certain conditions could cause clarification of tomato juice or reduce the viscosity of tomato pulp *(28, 31, 32)*. Pectin-polygalacturonase (PG) of

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fungal origin has been widely used to clarify juices; however, according to Kertesz *(26)* it is questionable whether PG occurs naturally in most higher plants. Averrill *(5)* stated that PG presumably had no effect on clarification of citrus juices. He concluded that clarification was apparently due to partially demethylated pectic substances precipitating rather than to hydrolysis of the polygalacturonide chain. Joslyn, Mist, and Lambert *(21)* have recently demonstrated that PG is probably present in apples. Two additional depolymerizing enzymes were recently described by Seegmiller and Jansen *(39)* and by Roboz, Barrett, and Tatum *(36)*. Both of these enzymes, respectively named polymethylgalacturonase (PMG) and pectin depolymerase, can hydrolyze the methylated pectic chain—unlike PG and PD.

The thermal inactivation of pectic enzymes was studied by Joslyn and Marsh *(20)* who showed that it was necessary to heat orange juice 2 minutes at 87.5°C.(189.5°F.) or 8 minutes at 85°C.(185°F.) to inactivate pectic enzymes. Kertesz *(25)* demonstrated that tomato PE was inactivated when heated 45 seconds at 80°C.(176°F.). McColloch and Kertesz *(30)* reported that PE of tobacco and tomatoes heated one hour at 55°C. (131°F.) showed no loss of activity. When the tomatoes were heated at 60°C.(140°F.), gradual inactivation occurred; for rapid inactivation, as is required in commercially processing tomato juice, the tomatoes had to be heated above 80°C.(176°F.). According to Braverman *(13)* pectic enzymes in citrus juices are inactivated by heating the juice one minute at 88°C. (190°F.) or 4 minutes at 85°C.(185°F.). The reports on the thermal resistance of PE thus indicate that considerable variation exists in the heat stability of PE in different products.

It has been suggested that only about 50% of the activity of pectic enzymes in citrus products is inactivated by the pasteurization procedures used commercially at present *(3)*.

EXPERIMENTAL

In this investigation, 5 ml. of single-strength grapefruit juice were put into glass tubes, 9 mm. in outside diameter, and sealed. The tubes were submerged in a constant-temperature water bath for the desired lengths of time in accordance with methods developed for microbial studies *(10, 12, 16, 23, 41)*. The tubes were immediately cooled in an ice-water bath at the end of the heating periods. The heating and cooling rates of the contents of the tubes were determined using thermocouples so that the heat lag could be calculated by the Ball formula method *(1, 8, 9)* and deducted from the actual heating period to yield the equivalent time at each temperature used. The grapefruit juice used was reamed by hand and strained through 2 layers of cheesecloth to remove part of the pulp.

To determine survival-destruction points PE activity was determined by the qualitative method of Kertesz *(24)*. This method consisted of adding 1.0 ml. of the juice to 25 ml. of a 1.0% pectin solution in the presence of methyl red. The solution was titrated with alkali until no pink color remained and then allowed to stand 10 minutes at 30°C.(88°F.). If the solution turned red within 10 minutes, the test was recorded as positive. The thermal inactivation times were determined on 11 batches of juice. Determinations were made at 90°, 85°, 80°, and 75°C.(194°, 185°, 176°, and 167°F.) using 10 tubes for each time-temperature combination. The time intervals ranged from 15 to 3 seconds, depending upon the temperature at which determinations were being made and the results of previous determinations; generally 6 periods of heating were used at each temperature.

PE activity was also determined quantitatively. The McColloch and Kertesz *(29)* modification and the General Foods Laboratory *(2)* modification of the method devised by Kertesz *(24)* were used. In the former method, 50 ml. of a 1.0% pectin solution (the pectin containing at least 8% methyl-ester groups) was made 0.1 N with respect to NaCl and then titrated to pH 7.0 with a standard alkali solution. Twenty ml. of the juice to be tested were added. The solution was maintained at 30°C.(86°F.) and constantly stirred and adjusted to the original pH. After 30 minutes the solution was titrated to pH 7.5. A blank was run on juice that had been boiled. The difference in the amount of alkali required for the blank and the sample was considered to be due to PE activity. The points of difference between this method and the General Foods method were that in the General Foods method the pH of the pectin solution containing the juice was adjusted to a pH value in the range 7.5-9.0 and the alkali was then added in 2-ml. portions after the pH had fallen to 7.5.

The thermal inactivation rates of PE were measured at 90°C.(194°F.) and 80°C. (176°F.). Because 20 ml. of juice were required to measure PE activity by either of the quantitative methods, 4 tubes from each time-temperature combination were pooled for each determination. A minimum of 10 measurements was made for each time-temperature combination for each batch of juice. The PE inactivation rates of 10 batches of juice were determined.

According to Kertesz *(26)* the methyl ester content of the pectin used in the determination of pectinesterase activity should be above 8%; therefore, the methyl ester content of all pectin used was determined to see whether or not it was suitable for such determinations. The methyl ester content of the pectins used varied from 8.2 to 14.3%.

Two methods were employed to determine the activity of pectic depolymerase.[c] One method consisted of the addition of 25 ml. of juice to be tested to 50 ml. of a 0.5% pectin solution. The resulting solution was then incubated 24 hours at 40°C. (104°F.). At the end of the incubation period, the calcium pectate method of Carré and Haynes *(14)* was used to measure the amount of non-degraded pectin. The other method was a procedure devised by Bell, Etchells, and Jones *(11)*. This method is based on measuring the loss in viscosity of a pectin solution that has been acted on by PG. The apparatus used was modified slightly from that described by Bell *et al. (11)* in that the viscosimetric pipette was jacketed in a glass tube through which water at a temperature of 30°C.(86°F.) was passed to hold the temperature constant. A 3% pectin solution, buffered at pH 4.0 with a sodium citrate-citric acid, was used as the

[c]The term pectic depolymerase is used throughout this paper to designate all pectin and/or pectic acid depolymerizing enzymes.

TABLE 1

F_{180} and z values of pectinesterase of grapefruit juice

Batch number	pH	F_{180}	z
	Unbuffered		
1	3.21	2.50	26.8
2	3.31	2.50	26.8
3	3.31	2.21	25.6
4	3.48	2.84	27.6
5	3.40	2.65	27.2
	Buffered pH 3.2		
6	3.20	2.26	26.4
7	3.20	2.35	25.3
8	3.20	2.51	26.3
	Buffered pH 3.4		
9	3.40	2.86	28.5
10	3.40	2.35	28.3
11	3.40	3.02	28.3

TABLE 2

Pectinesterase activity of grapefruit juice that had been heat treated various lengths of time at 90°C.(194°F.)

Time heated in minutes (lag deducted)	Pectinesterase activity PE μ ($\times 10^{-4}$)									
	Batch number									
	1	2	3	4	5	6	7	8	9	10
pH	3.25	3.28	3.30	3.28	3.31	3.35	3.38	3.21	3.32	3.27
0.00	26.7	28.5	34.4	24.5	31.1	20.2	31.2	19.2	32.5	21.5
0.02	25.2	26.6	31.3	24.2	30.2	18.1	28.2	17.0	27.9	19.9
0.11	17.5	18.7	22.2	15.6	16.3	16.2	21.4	15.0	21.8	17.8
0.24	11.6	13.8	19.4	9.1	12.4	11.1	14.1	9.2	15.2	9.9
0.51	4.1	9.6	10.5	3.0	8.1	9.1	10.1	2.5	11.8	9.2
0.65	0.0	5.1	6.1	0.0	2.2	3.2	2.5	0.0	4.5	3.8
0.88	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

TABLE 3

Pectinesterase activity of grapefruit juice that had been heat treated various lengths of time at 80°C.(176°F.)

Time heated in minutes (lag deducted)	Pectinesterase activity PE μ ($\times 10^{-4}$)									
	Batch number									
	1	2	3	4	5	6	7	8	9	10
pH	3.25	3.28	3.30	3.28	3.31	3.35	3.38	3.21	3.32	3.27
0.00	26.7	28.5	34.4	24.5	31.1	20.2	31.2	19.2	32.5	21.5
0.08	26.6	27.2	32.5	24.1	29.3	19.2	28.3	18.2	29.0	20.1
0.37	20.2	23.4	29.8	22.2	25.2	17.8	24.5	16.5	25.2	17.9
0.81	17.2	20.5	25.4	19.7	23.1	15.9	20.1	13.8	21.1	16.0
1.31	16.1	18.9	23.3	16.6	20.4	13.4	17.5	11.4	18.4	13.7
1.81	15.4	16.2	20.2	15.5	19.5	11.2	13.8	9.8	15.2	11.4
2.31	14.8	14.7	15.1	12.2	14.8	8.2	11.2	7.2	11.8	8.6
2.81	10.9	11.6	12.2	8.8	13.3	6.1	6.8	5.0	4.1	7.0
3.31	5.1	7.4	6.1	1.6	5.9	3.5	4.2	1.2	4.9	6.0
3.56	2.2	3.1	5.3	0.0	4.1	2.1	2.1	0.0	0.9	3.2
3.81	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

substrate. It was strained through cheesecloth to insure uniformity. To 25 ml. of the pectin solution, 5.0 ml. of the juice were added and the solution incubated 48 hours at 40°C.(104°F.). Blanks were run on juice that had been boiled and the difference in viscosity was used to calculate enzyme activity.

RESULTS

Thermal inactivation points of PE at 90°C.(194°F.) ranged from 0.65 to 1.06 minutes (lag deducted) for the 11 batches of juice tested. The inactivation times at 85°, 80°, and 75°C.(185°, 176°, and 167°F.) ranged, respectively, from 1.32-1.98, 3.40-4.13, and 7.48-9.82 minutes. The pH of the samples used in these determinations varied from 3.20 to 3.48. The lower destruction times and lower z values (the number of degrees Fahrenheit required for the thermal destruction time curve to pass through one log cycle) tended to occur at the lower pH levels.

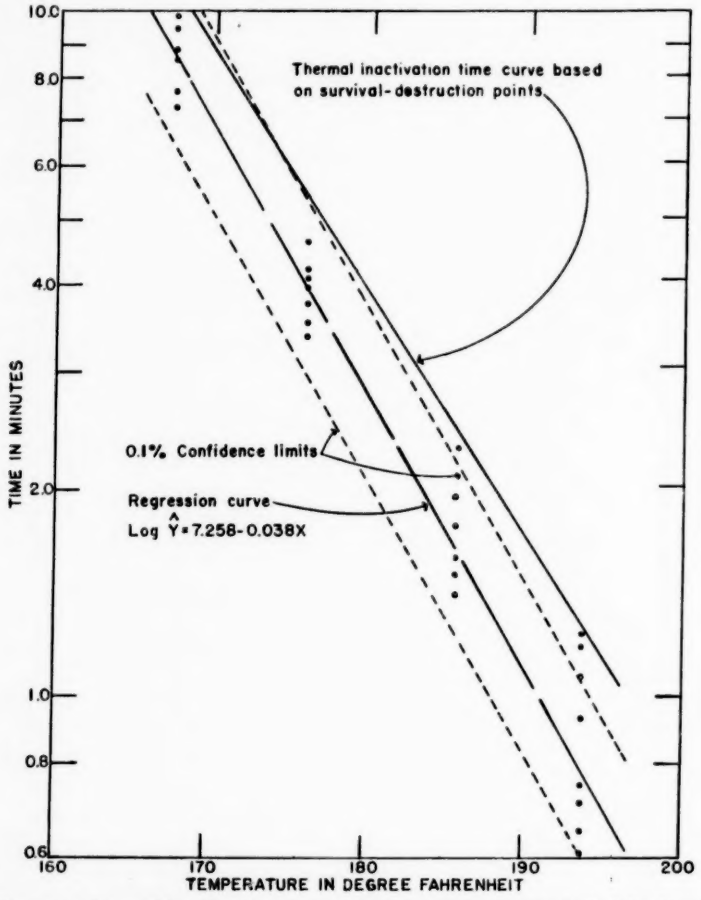

Figure 1. Thermal-inactivation curve of pectinesterase of grapefruit juice based on maximum survival points from survival-destruction determinations and regression curve of all survival-destruction points with 0.1% confidence limits. Points shown are the maximum survival points for each batch.

From the survival-destruction points, thermal inactivation time curves were plotted for each batch of juice. F_{180} (the destruction time in minutes at 180°F.) was used as a reference point of enzyme destruction. The F_{180} and z values for the 11 batches are shown in Table 1. The composite regression curve of thermal inactivation times with 0.1% confidence limits *(18)* is shown in Figure 1. The calculated regression curve gave an F_{180} value of 2.62 and a z value of 26.1. The upper confidence limit *(18)* at the 0.1% level for the F_{180} value was 3.12. The maximum F_{180} value actually observed was above this limit.

Thermal inactivation rate studies yielded F_{180} values comparable to those observed using the survival-destruction criterion. Thermal inactivation rates were measured because under some conditions of handling and storage complete destruction of the enzyme might not be necessary to prevent gelation or clarification during the usual marketing period. The thermal inactivation rates are shown in Tables 2 and 3.

Enzymes which depolymerized pectin and/or pectic acid was demonstrated in 4 out of 14 batches of grapefruit examined. Whenever activity was demonstrated it was slight and it was demonstrated by the method of Bell *et al. (11)* rather than by the Carré and

TABLE 4

Pectic depolymerase[c] activity of grapefruit juice that had been heat treated various lengths of time at 90°C.(194°F.)

Time heated in minutes (lag deducted)	Percent activity			
	Batch number of juice			
	1	2	3	4
pH	3.30	3.35	3.40	3.26
0.00	100.0	100.0	100.0	100.0
0.02	91.2	80.9	82.3	92.4
0.11	88.4	71.1	70.9	88.3
0.24	68.7	61.8	54.8	71.0
0.51	58.5	54.4	49.4	57.9
0.65	42.9	39.2	40.3	42.1
0.88	23.1	21.1	19.4	12.4
1.14	6.0	7.8	6.9	0.0
1.39	0.0	0.0	0.0	0.0

TABLE 5

Pectic depolymerase[c] activity of grapefruit juice that had been heat treated various lengths of time at 80°C.(176°F.)

Time heated in minutes (lag deducted)	Percent activity			
	Batch of juice			
	1	2	3	4
pH	3.30	3.35	3.40	3.26
0.00	100.0	100.0	100.0	100.0
0.08	89.4	90.1	84.2	86.7
0.37	71.2	70.2	69.8	64.5
0.81	60.5	62.3	61.2	59.2
1.31	50.1	49.8	46.7	45.6
1.81	39.2	37.2	35.2	33.5
2.31	24.8	24.6	21.3	18.9
2.81	14.2	15.8	13.8	12.2
3.31	8.2	9.2	7.6	6.5
3.56	4.1	5.6	4.7	3.2
3.81	3.5	3.8	2.0	1.9
4.06	0.0	2.4	0.0	0.0
4.31	0.0	0.0	0.0	0.0

Haynes *(14)* method. The thermal inactivation rates at 90°C.(194°F.) and 80°C.(176°F.) of pectic depolymerase are shown in Tables 4 and 5. The composite regression curve of the thermal inactivation times of the 4 batches and the 1.0% confidence limits *(18)* are shown in Figure 2. The upper confidence limit *(18)* at the 1.0% level for F_{180} was 4.00. All values actually observed were below this value.

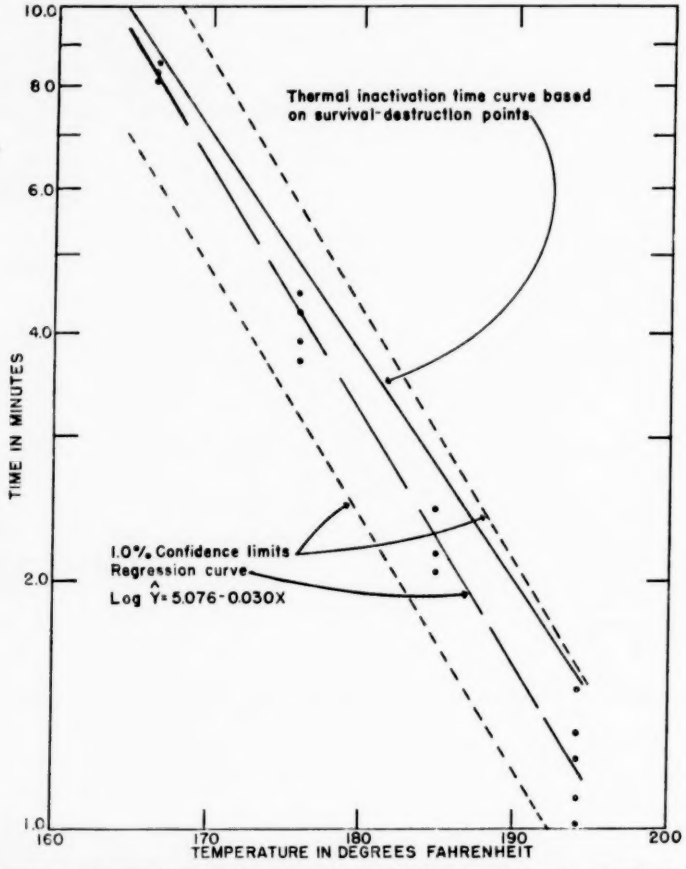

Figure 2. Thermal-inactivation curve of pectic depolymerase* of grapefruit juice based on maximum survival points from survival-destruction determinations and regression curve of all survival-destruction points with 1.0% confidence limits. Points shown are the maximum survival points for each batch.

DISCUSSION

Thermal destruction times of PE reported herein are in the same range as those reported by previous investigators *(4, 13, 38)*. The thermal inactivation times at 90°C.(194°F.) were somewhat higher than those reported by Atkins *et al. (4)* whereas at 80°C.(176°F.) they were lower; thus the slope of the inactivation curve differs from that of Atkins *et al. (4)*. They

reported their work in PE activity per degree Brix while the work reported here is in PE activity per ml. The inactivation times for pectin enzymes reported by Braverman *(13)* at 88°C.(190°F.) and 85°C.(185°F.) were slightly higher than the corresponding values taken from the thermal destruction curves of this investigation.

When this investigation was initiated, the decrease in viscosity of a pectin substrate was considered indicative of the action of PG *(11, 26, 35)*. Recently, Seegmiller and Jansen *(39)* and Roboz *et al. (36)* have reported that PMG and pectin depolymerase can split pectin directly (without prior demethylation, unlike PG) which action causes a decrease in viscosity; consequently viscosimetric measurements must now be considered of limited value unless it is known whether the pectic substrate is or is not methylated when depolymerization occurs. Because the authors were thinking in terms of PG, or possibly PD, they considered failure to observe loss in viscosity during the early tests possibly due to insufficient PE to demethylate the pectin *(19)*. To avoid any such deficiency, PE was added to all substrates thereafter. As a result, it is impossible to state whether the depolymerizing action later observed arose from a depolymerizing enzyme similar to PG or to PMG. The pH at which the reaction was carried out was nearer the optimum pH for PG than for PMG or pectin depolymerase; the fact that no positive tests were noted prior to adding PE routinely to the substrate, and the finding of Joslyn *et al. (21)* that PG appears to occur naturally in apple juice indicates that the depolymerizing enzyme was probably PG or PD. However, the rate of decrease in viscosity did not appear to be uniform; thus the possibility exists that the depolymerizing action observed may have been caused by two enzymes. Every effort was made during the tests to prevent microbial contamination; thus it appears that the depolymerization observed arose from the action of a depolymerizing enzyme naturally present in grapefruit juice. Inasmuch as the depolymerizing enzyme, or enzymes, were more heat resistant than PE, they might, under certain conditions, be involved in clarification of citrus juices or concentrates.

SUMMARY

Thermal destruction times of pectinesterase of grapefruit juice were determined at 90°C.(194°F.), 85°C.(185°F.), 80°C.(176°F.), and 75°C.(167°F.). The thermal destruction times were 1.06, 1.98, 4.13, and 9.82 minutes, respectively, at the 4 temperatures.

The F_{180} and z values were calculated for pectinesterase. The F_{180} values varied from 2.21 to 3.35 minutes. The variation in z values was from 25.3 to 28.5.

The thermal destruction times of the pectin or pectic acid depolymerizing enzyme of grapefruit juice were determined at 90°C.(194°F.), 85°C.(185°F.), 80°C.(176°F.), and 75°C.(167°F.). The thermal destruction times were 1.39, 2.06, 4.23, and 8.49 minutes, respectively, at the 4 temperatures.

The F_{180} and z values were calculated for pectic depolymerase.[c] The F_{180} values varied from 2.35 to 3.45 minutes. The variation in z values was from 35.2 to 41.2.

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ENZYME AND ASCORBIC ACID CONTENT OF FRESH AND FROZEN PINEAPPLE[a]

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Aside from studies of the proteolytic enzyme, bromelin, the literature contains no investigation of enzymes present in fresh pineapple. This study was undertaken to determine which enzymes are present in fresh pineapple; since pineapple is a good source of ascorbic acid, particular emphasis was given to enzymes which destroy this vitamin. Also investigated were changes in enzyme and vitamin levels during home freezing and freezer storage of fresh pineapple packed by various methods.

The 3 enzymes which commonly catalyze the oxidation of ascorbic acid in fruits and vegetables are peroxidase, phenolase, and ascorbase. Qualitative tests for the presence of each were made. Aside from the enzymes known to destroy ascorbic acid, qualitative determinations were made of the presence of several dehydrogenases that may or may not be involved in the loss of this vitamin. And finally, in the case of phosphatase, which is unrelated to ascorbic acid oxidation, quantitative measurements were made of its activity.

MATERIALS AND METHODS

Sun-ripened Natal pineapples were sent by express at definite intervals from Miami Plantation in Miami, Florida. The fruit was analyzed as soon as possible after delivery for quantitative determinations of enzyme activity and ascorbic acid content; for other experiments which could not be done within 3 days after receiving shipments, the whole pineapples were stored in a freezer at −20°C. until they could be assayed.

For both vitamin and enzyme assays, a sample consisted of radial longitudinal wedges of approximately 50 g. each from 3 different pineapples. The wedges were pared, cored, and diced before weighed amounts of the extracting liquids were added. The sample was comminuted for 3 min. in a blender containing approximately 300 g. of extractant; a second blend using a 100-g. aliquot and 100 g. of extractant was comminuted for 1 min. Three percent and 0.4% oxalic acid solutions were the extractants for the first and second blends, respectively, in the ascorbic acid analyses. A 2% sodium chloride solution (+2.5 g. calcium carbonate in the first blend for peroxidase) was the extractant for all enzyme determinations. The extracts were filtered in a refrigerator at 2°C. Filtrates were analyzed within 1 hr. after filtration was completed. All solutions were made with water redistilled from glass apparatus.

Moisture content. A thin slice from a pared, radial, longitudinal wedge was cut transversely into three pieces, each of which constituted a sample. The total number of samples was 75 and this included equal quantities of center, crown, and base sections from various pineapples. The samples were dried in an air oven at 75°C. for 3 hrs., then in vacuo at the same temperature and 25-mm. pressure until the sample weights were constant to within 3 mg.

Ascorbic acid. The ascorbic acid content was measured spectrophotometrically, using 2,6-dichlorophenolindophenol.

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Peroxidase. The method of Sumner and Gjessing *(8)* was used for the determination of peroxidase activity. The unit of enzyme activity reported is the Purpurogallin Zahl (P.Z.), which is defined as grams of purpurogallin formed in 5 min. by 1 g. of fresh pineapple at 30°C., pH 5.9, in the presence of excess hydrogen peroxide.

The concentration of hydrogen peroxide for optimum pineapple peroxidase activity was determined over the range 0.001-10%, concentration of reagent. Milligrams of purpurogallin formed per unit time were determined at each concentration of hydrogen peroxide solution, and activity was converted to "percent of maximum activity."

The thermal destruction time for pineapple peroxidase was determined at temperatures ranging from 78°-121°C. For temperatures lower than 100°C., empty test tubes (6 × 100 mm.) were put in an oil bath which was placed in a constant-temperature water bath until temperature equilibrium was reached. One ml. of crude enzyme extract was introduced via capillary into the bottom of one of the tubes and after a definite time interval, the tube was removed and its contents poured immediately into a test tube (15½ × 2 cm.) which contained 1 ml. 1% hydrogen peroxide, 1 ml. 0.04 *M.* guaiacol and a small amount of crushed ice, made with redistilled water. For the 100°C. determination, a beaker of boiling water was used, and for 121°C., a pressure saucepan.

The effects of various salts as inhibitors and catalysts of pineapple peroxidase were observed. Solutions were made of ten different salts in molar concentrations of 10^{-3}, 10^{-4}, and 10^{-7}. Peroxidase activity in the presence of the various salts was determined by the rapid colorimetric method of Ponting and Joslyn *(6)*. The percent transmission of light in a Coleman Universal Spectrophotometer at 425 mμ. was recorded at 15-sec. intervals over a 5-min. period after addition of the enzyme. The change in log T/minute was determined for each salt at each concentration. At regular intervals the light transmission in cuvettes containing redistilled water instead of a salt solution was read in order to detect any decrease in peroxidase activity independent of that caused by the various salts.

Polyphenolases. Several phenolic substrates including mono- and diamino compounds, mono-, di-, and trihydroxyphenols, and one monoaminohydroxy compound were dropped on freshly-cut surfaces of fresh pineapple to test qualitatively for the presence of phenolase *(7, 2, 1)*.

Ascorbase. The method of Ponting and Joslyn *(6)* was used to determine whether ascorbase was present in fresh pineapple.

Dehydrogenases. The Thunberg technique was used for the qualitative determination of dehydrogenases *(9)*.

Phosphatase. Quantitative measurements of phosphatase were made according to the procedure of Morris, Weast, and Lineweaver *(5)*. Maximum light absorption by the phenol salt, using the Coleman Universal Spectrophotometer, was found to occur between 700-710 mμ. A unit of phosphatase activity is defined as the milligrams of phenol liberated by 1 g. of fresh pineapple from excess disodium phenylphosphate as a result of hydrolysis for 10 min. at 47.5°C., pH 5.4.

Home-freezing experiments. Approximately 100-g. portions of pared, cored, and cubed pineapple were put in Vapocans. The cubed pineapple was sirup-packed, sugar-packed, and sugarless-packed according to the procedures of Joslyn and Hohl *(3)*.

Analyses for ascorbic acid content and P.Z. were made after freezing overnight at −20°C., after freezer storage for 2 mos., and after freezer storage for 4 mos. For all assays the entire contents of the Vapocan were used as one sample.

RESULTS

The average moisture content of pineapples was found to be 81.2%, standard deviation 2.1.

The ascorbic acid content and enzyme activity of fresh pineapple are recorded in Table 1. The results show that Natal pineapples contain about 23.5 mg. ascorbic acid/100 g. fruit. Since this is about one-third the recommended allowance of the vitamin, Natal pineapples may be considered as a good source of ascorbic acid. One g. of fresh pineapple contains sufficient peroxidase to catalyze the formation of 0.0043 g. of purpurogallin

TABLE 1

Level of ascorbic acid and peroxidase in fresh Natal pineapples

Sample[d]	Ascorbic acid (mg./100 g. fruit)	P. Z. (g. purpurogallin/g. fruit)
1	23.8	0.0045
2	21.2	0.0042
3	23.2	0.0043
4	25.8	0.0042
5	25.0	0.0046
6	23.5	0.0037
7	21.7	0.0043
Mean	23.5	0.0043
Standard deviation	1.6	0.0003

[d]Each sample consists of wedges from three pineapples.

from pyrogallol in the presence of excess hydrogen peroxide as substrate during a 5-min. reaction period at 30°C.

Ascorbic acid filtrates were placed in the freezer at −20°C. and analyzed periodically in order to find out whether the vitamin was stable in 3% oxalic acid as extractant after storage for short periods of time at a low temperature. Mean values of the ascorbic acid content of the filtrates were 8.8 mg./100 g., 9.0 mg./100 g., and 9.6 mg./100 g. of fresh pineapple after storage periods of 0 days, 5 days, and 4 wks. By analysis of variance,[f] an F value of 1.36 is obtained, indicating that no significant change in the ascorbic acid content of the filtrates occurred during a storage period of 4 wks. at −20°C.

The concentrations of hydrogen peroxide reported in Table 2 are based on the titration of Merck's Superoxol with standard $KMnO_4$. The hydrogen peroxide concentration of optimum peroxidase activity was 1%, or 0.3 *M.*, which corresponds to a concentration of 0.025% in the reaction mixture.

[f]As proposed by Fisher *(4)*.

TABLE 2

Effect of H_2O_2 concentration on activity of peroxidase

Concentration H_2O_2* (%)	Peroxidase activity in percent of maximum activity
0.001	16
0.01	20
0.1	48
0.5	70
0.6	78
0.7	86
0.9	94
1.0	100
2.0	92
3.0	93
4.0	88
5.0	86
10.0	76

*Refers to concentration of H_2O_2 in reagent solution before it is added to the reaction mixture. Total volume of reaction mixture in all cases was 40 ml.

The activities indicated in Figure 1 were determined with frozen pineapples which had been stored whole. The optimum activity occurred at 40°C.; at temperatures above 40° there was rapid heat inactivation of the enzyme; and at 82°, no activity was evident.

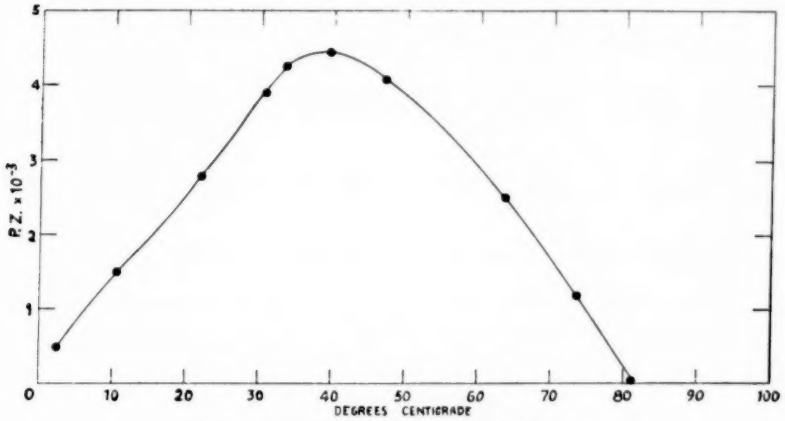

Figure 1. Variation of P. Z. with temperature.

The thermal destruction time of pineapple peroxidase at various temperatures is shown in Table 3. These data support the frequently reported fact that peroxidase is less sensitive to high temperatures than many other enzymes. Although 24 seconds at 121°C. is the thermal destruction time for peroxidase, the crude extracts used in "blanks" for the determination of enzyme activities were held 7 min. at this temperature for these reasons: the extract was contained in a 50-ml. flask rather than a small test tube; a larger quantity of extract was to be inactivated; the flask had not previously been brought to temperature equilibrium; and, since peroxidase activity frequently returns in extracts that are allowed to stand for short periods of time, the additional heating insured no return of activity for ½ hr. or longer.

The salt concentrations used in studying catalytic and inhibitory effects on peroxidase are shown in Table 4. These concentrations refer to molarity before the salt solution was added to the contents of the reaction tube. At the 10^{-1} *M.* concentrations, cyanide, azide, sulfide, calcium, and fluoride

TABLE 3

Thermal destruction time of pineapple peroxidase

Temperature (°C.)	Thermal destruction time (min.)
78	8.0
84	3.5
94	1.5
100	0.5
121	0.4

TABLE 4

Comparative effects of several salts at various concentrations on pineapple peroxidase activity

Salt	Percent change in reaction rate		
	10^{-3} molar	10^{-4} molar	10^{-7} molar
KCN	−100	− 9	+3
NaN_3	−100	−14	+3
$FeCl_3$	.....[a]	−31	+3
$CuCl_2$	.....[a]	+ 7	+1
Na_2S	−100	+ 7	+1
NaBr	− 6	+ 3	+8
$CaCl_2$	− 13	+14	+1
$MgCl_2$	+ 2	+ 3	+7
NaF	− 26	− 2	+1
NaCl	+ 4	+ 4	+1

[a] Colored product other than the usual oxidation product of guaiacol was formed, so that the initial transmission of light (15 seconds after addition of enzyme) was 5% or less.

ions were inhibitors; sodium bromide was a weak inhibitor, while magnesium chloride and sodium chloride showed no inhibitory effects. At 10^{-4} *M.* concentrations, azide and ferric ions were inhibitors; cyanide was a weak inhibitor; the other salts showed slight inhibitory or catalytic effects. None of the salts showed inhibitory effects at 10^{-7} *M.* concentration.

All qualitative tests to show the presence of polyphenolases were negative.

Results based upon Ponting and Joslyn's method *(6)* for the determination of ascorbase showed the absence of this enzyme from fresh pineapple.

Positive dehydrogenase tests were obtained with lactic, malic, citric, succinic, formic, and oxalic acids, and with dextrose, choline, and ethyl alcohol. Negative results were secured with formaldehyde and hydroquinone.

Fresh Natal pineapple was found to have a phosphatase activity of 3.0 units.

Analysis of variance was used to determine whether the ascorbic acid content of frozen pineapple was significantly affected by the methods of packing and length of storage. The variance ratios for ascorbic acid content as shown in Table 5 indicate that the variation among packs is greater than the variation among periods of storage, but that the ascorbic acid content does not vary significantly among packs or among periods of storage. Therefore, fresh pineapple retained its ascorbic acid to the same extent when packed by 3 different methods, and the vitamin content of all pineapple samples remained unchanged over a 4 months' storage period at −20°C. It has already been shown that ascorbic acid is not affected by the freezing process *(10)*; hence, measurements of the vitamin content after packing but before freezing are not reported here.

While freezing experiments showed no differences in ascorbic acid content among the packs, differences among them were demonstrated at a refrigerator temperature of 2°C. Five Vapocans were packed by each of the 3 methods and stored overnight in the refrigerator (data not reported). These were assayed the following morning for ascorbic acid. The sugarless-packs contained a mean ascorbic acid content of 8.9 mg. ± 1.8/100 g., the sugar-packs 8.8 mg. ± 1.7/100 g., and sirup-packs 11.3 mg. ± 1.6/100 g., calculated to the fresh weight basis. Twenty-two percent of the ascorbic

TABLE 5

Levels of ascorbic acid and peroxidase in pineapples prepared by three packing methods and stored in freezer for varying lengths of time

Pack	Ascorbic acid (mg./100 g.)			P. Z.		
	After freezing	After 2 months' storage at −20°C.	After 4 months' storage at −20°C.	After freezing	After 2 months' storage at −20°C.	After 4 months' storage at −20°C.
Sugarless						
Mean[b]	11.2	11.5	10.1	0.0041	0.0040	0.0041
Standard deviation	3.0	2.2	2.0	0.0005	0.0003	0.0011
Sugar						
Mean[b]	10.7	9.9	10.4	0.0040	0.0036	0.0031
Standard deviation	2.4	2.6	1.0	0.0010	0.0005	0.0009
Sirup						
Mean[b]	10.6	11.3	11.6	0.0040	0.0035	0.0033
Standard deviation	1.2	1.4	1.7	0.0009	0.0008	0.0007

	Source of variation	$F_{a.a.}$[1]	$F_{p.z.}$[2]	Significance
Analysis of variance	Packs (P)	0.313	0.942	Not significant
	Replication within P			
	Storage times (S)	0.056	1.598	Not significant
	S × P	0.959	0.444	Not significant
	Residual			

[b] Five replicates included.
[1] Variance ratio-ascorbic acid.
[2] Variance ratio-purpurogallin Zahl.

acid was lost when sugarless- or sugar-packed pineapple was left overnight in the refrigerator, whereas the sirup-packed pineapple had the same vitamin content as that of frozen. These results indicate that a delay in freezing would have less effect on the ascorbic acid content of sirup-packed than of either sugar- or sugarless-packed pineapple; also shown is the fact that a delay in freezing has more effect than has a 4 months' storage period in the freezer.

Analysis of variance was used to determine whether the peroxidase activity of frozen pineapple was significantly affected by methods of packing and length of storage. The variance ratios for enzyme activity in Table 5 show that the F value for periods of storage is greater than the F value for packs, but that neither the variation among packs nor the variation among periods of storage is significant. These findings support the hypothesis that the peroxidase activity of pineapple packed by these 3 methods and stored at −20°C. is unchanged over a period of 4 months.

SUMMARY

Peroxidase was present in pineapple and had an activity of 0.0043 g. purpurogallin ± 0.0003/g. fresh pineapple/5 min. at 30°C., pH 5.9. Phenolase and ascorbase were not found in fresh pineapple. The presence of several dehydrogenases was shown by qualitative tests. Phosphatase activity of 3 mg. phenol/g. pineapple/10 min. at 47.5°C. and pH 5.4 was observed.

The fact was confirmed that fresh pineapples are a good source of ascorbic acid, the content of Natal pineapples being 23.5 mg. ± 1.6/100 g. fruit.

The ascorbic acid content of frozen pineapple was constant over a 4 months' storage period at −20°C., regardless of whether the sugarless-, sugar-, or sirup-pack method was used. Results showed that 22% of the ascorbic acid in sugarless- and sugar-packed pineapple was lost if the freshly-packed containers were put in the refrigerator overnight; this indicated that fruit prepared by these methods should be placed in the freezer as soon as possible to prevent a loss of the vitamin.

No change in the peroxidase activity was shown when pineapple packed by the 3 methods was stored 4 months at −20°C.

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A RAPID METHOD FOR ESTIMATING PECTIC SUBSTANCES IN CITRUS JUICES[a,b,c]

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Citrus processors are becoming increasingly aware of the importance of pectic substances in frozen citrus juices. Pectic substances are naturally occurring colloidal stabilizers that give citrus juices a viscosity or consistency often termed "body" by the fruit juice industry. When a citrus juice lacks these colloidal pectic substances the suspended cellular material settles rapidly and the juice is clear rather than cloudy. These thin-bodied juices are commonly termed "watery."

Recently pectic substances became of vital interest to producers of frozen citrus concentrates when the problems of gelation and clarification were found by Rouse *(9)* to be related to the low-methoxyl pectin content. Gelation and clarification of frozen citrus concentrate are 2 related defects resulting from demethylation of pectin by the enzyme, pectinesterase.

The importance of pectic substances to the quality of frozen citrus concentrates made it desirable to develop a rapid method for determining the type and quantity of pectic substances in these products. Basic investigational work on pectic substances and laboratory control procedures in concentrating plants have been hampered previously by the tedious nature of standard pectin analyses. The Carré and Haynes *(3)* calcium pectate method and the A.O.A.C. *(1)* pectic acid method are long analyses that require careful attention to technique. The acetone precipitate method of Hinton *(6)* and the pectic acid precipitate method of Fellers and Rice *(5)* are simplified methods that give comparative values for samples within a series, but are not sufficiently accurate for determining the pectic content of citrus juices.

Recently pectic substances in citrus have been determined by optical rotation *(8)* and the pectic substances in cotton have been determined by a colorimetric reaction with carbazole *(10)*. The latter method was applied by Averill *(2)* to frozen citrus concentrate. Further modification of the colorimetric procedure led to the development of the following method. The new method is rapid and well suited for routine laboratory analyses.

The colorimetric determination of pectic substances is based on Dische's *(4)* carbazole-hexuronic acid-sulfuric acid reaction. Depending upon the type of standard, results may be expressed as anhydrogalacturonic acid, pectic acid, or in terms of a stated pectin or calcium pectate. It is desirable that the results be reported as anhydrogalacturonic acid, since this is the basic structural unit of the pectin molecule.

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[b]Cooperative research project of Florida Citrus Experiment Station and Florida Citrus Commission, aided by grant from the American Can Company.

[c]Florida Agricultural Experiment Station Journal Series, No. 78.

EXPERIMENTAL PROCEDURE

The pectic substances are divided into 3 fractions by progressive extractions with distilled water, 0.4% sodium hexametaphosphate and 0.05 N sodium hydroxide. All separations are made by centrifugation followed by decantation.

Purification and extraction procedures require approximately 3 hours and the colorimetric determination of anhydrogalacturonic acid takes about 20 minutes.

The precision of the colorimetric method is ± 1% and duplicate samples carried through the extraction and colorimetric procedures should agree within 10%.

Reagents.

Ethyl alcohol, 95%.

Ethyl alcohol, ca 60%. Add 3 volumes of distilled water to 5 volumes of 95% ethyl alcohol.

Ethyl alcohol, purified. Reflux one liter of reagent grade 95% ethyl alcohol with 4 g. of zinc dust and 2 ml. of concentrated sulfuric acid for 15 hours and distill, using all-glass equipment. Redistill, using 4 g. of zinc dust and 4 g. of potassium hydroxide.

Sodium hexametaphosphate, technical, 0.4% solution.

Sodium hydroxide, 1 N.

Sodium hydroxide, 0.05 N.

Sulfuric acid, C.P.

Carbazole, 0.1% alcoholic solution. Recrystallize reagent grade carbazole from toluene. Dissolve 100 mg. of recrystallized carbazole in purified alcohol and dilute to 100 ml. with purified alcohol.

Galacturonic acid, monohydrate, reagent grade.

Extraction method. Blend the frozen concentrated citrus juice, ca 42° Brix for 3 minutes in a Waring blendor. Pipette 5 ml. of the blended juice into a tared 50-ml. graduated centrifuge tube and weigh on torsion balance to ± 0.005 g. Dilute to 10 ml. with distilled water and add 30 ml. of 95% ethyl alcohol.

For canned single-strength or fresh juice use a 20-ml. sample of blended juice, weigh, and precipitate the pectic material with 60 ml. of 95% ethyl alcohol in 100 ml. centrifuge tubes. This results in a 70% alcoholic mixture.

Heat the alcoholic mixture for 10 minutes in a water bath at 85°C., ± 2°C., with occasional stirring. The solution should gently boil during the last half of the heating period. Centrifuge the tubes at 2,100 r.p.m. (1,000 × gravity) for 10 minutes. Decant and discard the alcoholic solution.

Add 40 ml. of the 60% alcohol to the precipitate and mix thoroughly. Heat for 10 minutes in a water bath at 85°C., centrifuge, and decant as above.

Add 40 ml. of distilled water to each centrifuge tube and stir until the mixture is evenly dispersed. Allow the mixture to stand 10 minutes and stir again. Centrifuge as before and decant into a 100-ml. volumetric flask. Repeat the water extraction, using 40 ml. of distilled water, centrifuging and decanting into the same volumetric flask. Add 5 ml. of 1 N sodium hydroxide to the water extract and dilute to volume. Mix and let stand at least 15 minutes before beginning the colorimetric procedure.

To the residue add 40 ml. of 0.4% sodium hexametaphosphate solution, stir, and allow the solution to stand for 10 minutes at room temperature. Centrifuge as before and decant into a 100-ml. volumetric flask. Repeat the extraction, using 40 ml. of 0.4% sodium hexametaphosphate, centrifuge and decant into the same 100-ml. volumetric flask. Add 5 ml. of 1 N sodium hydroxide to the sodium hexametaphosphate extract and dilute to volume. Allow the solution to stand at least 15 minutes before beginning the colorimetric procedure.

Sodium hydroxide is added to the water and sodium hexametaphosphate extracts to saponify the pectins to sodium polygalacturonate. Thus, all 3 extractions contain pectic substances of similar composition and molecular size.

Extract the remaining residue with 40 ml. of 0.05 N sodium hydroxide for 15 minutes at room temperature. Stir occasionally. Centrifuge for 10 minutes as before, and decant the extract into a 100-ml. volumetric flask. Make to volume.

Analyze 1-ml. aliquots of each of the above extracts by the colorimetric procedure.

Colorimetric procedure. Pipette 1-ml. aliquots of the extract into each of 2 large test tubes (25 × 200 mm.) and add 0.5 ml. of the 0.1% alcoholic carbazole to the sample tube and 0.5 ml. of purified ethyl alcohol to the blank tube. A white flocculent precipitate will form in the sample tube.

Add 6 ml. of concentrated sulfuric acid to each of the tubes with constant agitation. A Machlett automatic burette is advantageous for adding the acid. Rubber stopper the tubes and allow the color to develop for approximately 10 minutes before transferring the solution to microabsorption cells. The 10-minute interval serves as a heating period (heat of dilution).

Exactly 15 minutes after adding the acid determine the percent transmittance in an electrophotometer with a 525 mμ filter, after nulling the instrument against the blank. Refer to the standard curve to obtain the concentration of anhydrogalacturonic acid (AGA). Calculate the percent of anhydrogalacturonic acid by the formula:

$$\% \text{ AGA} = \frac{\gamma \text{ AGA} \times \text{dilution volume (ml.)} \times 100}{1{,}000{,}000 \times \text{sample weight (grams)}}$$

Standard curve. Accurately weigh 120.5 mg. of galacturonic acid, monohydrate (vacuum dried 5 hours at 30°C.), and transfer to a liter volumetric flask. Add 10 ml. of 0.05 N sodium hydroxide and dilute to volume. Mix thoroughly and allow the solution to stand overnight. This standard solution contains 100 γ of anhydrogalacturonic acid per ml.

Prepare working standards covering the range of 10 to 70 γ of anhydrogalacturonic acid per ml. Develop the color standards as described for the sample solution, using 1-ml. aliquots of each working standard. Run 3 series of working standards and plot the log percent transmittance against concentration of anhydrogalacturonic acid in micrograms. This curve should be a straight line (Figure 1).

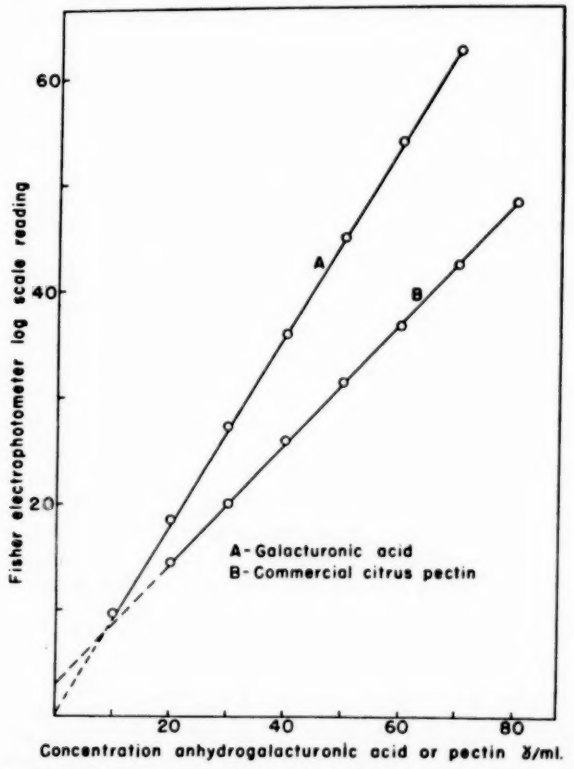

Figure 1. Standard curves for galacturonic acid and commercial citrus pectin.

Interfering substances. Impurities found in 95% ethyl alcohol may form strongly colored interference products when heated with carbazole. A 1-ml. sample of distilled water carried through the procedure should have a light transmittance greater than 98% when compared against a blank in which purified alcohol was substituted for the carbazole solution using a Fisher electrophotometer with a 525 mμ filter and a 3 ml. cylindrical microabsorption cell.

EXPERIMENTAL RESULTS

The standard curve for galacturonic acid had by extrapolation a zero intercept, as shown in Figure 1. The curve for commercial citrus pectin had a negative ordinate intercept characteristic of galacturonic acid-sugar mixtures. However, not all pectic substances had this characteristic intercept as a pectic extract of Duncan grapefruit showed a zero intercept. This depression of the line probably was caused by the presence of citric acid, since galacturonic acid-citric acid mixtures have positive ordinate intercepts.

No pectins could be purified to yield 100% anhydrogalacturonic acid, although a pectic acid approached this purity (Table 3).

The amount of pectic substances extracted by distilled water, sodium hexametaphosphate, and sodium hydroxide from a frozen orange concentrate of high pulp content and from a medium-pulp frozen grapefruit concentrate are shown in Table 1. Each of

TABLE 1

Pectic substances extracted from orange and grapefruit concentrates by successive extractions with distilled water, sodium hexametaphosphate and sodium hydroxide [d]

Extraction		Orange concentrate, 11.0% pulp		Grapefruit concentrate, 6.5% pulp	
Solution	No.	Anhydrogalacturonic acid	Total extracted AGA/group	Anhydrogalacturonic acid	Total extracted AGA/group
		mg./5 g.	%	*mg./5 g.*	%
Distilled water	1	0.912	87.1	0.624	60.6
	2	0.084	8.1	0.308	29.9
	3	0.022	2.1	0.072	7.0
	4	0.029	2.7	0.025	2.5
		1.047	100.0	1.029	100.0
0.4% sodium hexametaphosphate	1	5.920	87.0	1.960	90.6
	2	0.624	9.1	0.108	5.0
	3	0.142	2.1	0.084	3.9
	4	0.122	1.8	0.010	0.5
		6.808	100.0	2.162	100.0
0.05 N sodium hydroxide	1	5.000	92.8	0.248	96.9
	2	0.306	5.7	0.008	3.1
	3	0.082	1.5	0.000	0.0
		5.388	100.0	0.256	100.0

[d] Extractions made at 25°C. with 40-ml. portions of extractant using 5-g. samples of concentrate.

the four 10-minute water and sodium hexametaphosphate extractions was followed by a 10-minute centrifugal period. The sodium hydroxide extractions were for 15-minute periods followed by 10-minute centrifugations. In all cases over 90% of the extractable material was removed in the first 2 extractions by each solvent.

Sugars, citric acid, and ascorbic acid will react with carbazole to form colored products of varying degrees of intensity. At a wave length of 525 mμ (Beckman spectrophotometer, Model B) the maximum interference was caused by fructose (Figure 2). The interference caused by sucrose was of smaller magnitude, whereas only very small errors were introduced by citric acid, ascorbic acid, and glucose when present in amounts of 50 γ per ml.

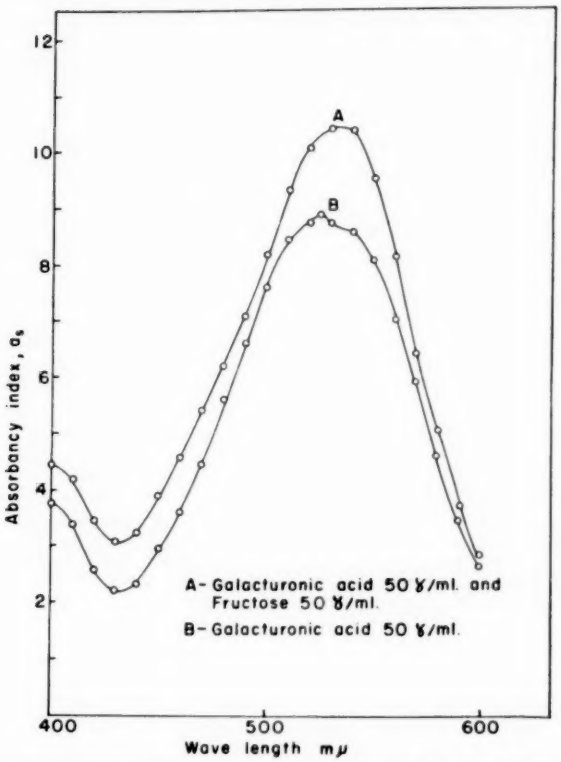

Figure 2. Absorbancy index of galacturonic acid and mixture of galacturonic acid and fructose.

The 2 alcoholic extractions with 70% and 60% ethyl alcohol were sufficient to reduce the concentration of these soluble juice constituents to a level of negligible interference, as indicated by no further improvement with subsequent alcohol extractions. At the same time the soluble pectic substances were precipitated from colloidal solution by the dehydrating action of the ethyl alcohol. The small amount of ethyl alcohol left in the precipitate following the second alcoholic extraction acts as a wetting agent and aids in dispersing the pectic substances in the following water extraction.

The maximum absorbancy for galacturonic acid and mixtures of galacturonic acid with citric acid, ascorbic acid, and glucose occurred at a wave length of 525 mμ. Maximum absorbancy for mixtures of galacturonic acid with sucrose and fructose occurred at a wave length of 535 mμ, as shown in Figure 2.

Comparison of methods. The pectic contents of 0.1% solutions of the alcohol precipitates of the component parts of Dancy tangerines were determined by both the Carré and Haynes *(3)* calcium pectate method and the colorimetric method described in this paper (Table 2). The calcium pectate samples from the Carré and Haynes analyses were dissolved in hot 0.5% ammonium oxalate and the anhydrogalacturonic acid contents of these ammonium pectate solutions were determined colorimetrically.

The anhydrogalacturonic acid contents of the original samples and calcium pectate samples are in good agreement for this type of determination, with the exception of the samples of rag and juice sacs. A longer period of saponification was probably necessary for the rag and juice sac samples, as indicated by the higher anhydrogalac-

TABLE 2

Pectic substances found in the alcohol precipitate of water extract of component parts of Dancy tangerines determined by both colorimetric and calcium pectate methods

Fruit part	Method		
	Calcium pectate	Colorimetric	
		Anhydrogalacturonic acid	
		Original sample	Calcium pectate[a]
	%	%	%
Flavedo	63.0	54.7	53.8
Albedo	62.4	57.2	57.5
Rag	61.8	45.4	53.8
Juice sacs	54.0	43.5	51.2
Seeds	15.6	18.6	17.5
Centrifuged juice	25.3	20.7	19.0

[a]Anhydrogalacturonic acid content of calcium pectate calculated as percent of original sample.

turonic acid contents found in the calcium pectate samples from the same pectic solutions.

The alcoholic precipitates of Duncan grapefruit and Pineapple orange concentrates were extracted with (a) sodium hexametaphosphate for 30 minutes at room temperature and (b) sodium hexametaphosphate followed by a hydrochloric acid extraction, both at 95°C. for 30 minutes. The latter 2 extracts were combined prior to analysis. The pectic substances were reprecipitated with 2 volumes of 95% ethyl alcohol, filtered, oven dried, and 0.1% solutions of the pectic extract were analyzed by the Carré and Haynes *(3)* calcium pectate method and by the colorimetric method (Table 3). All percentages reported are the average of duplicate samples.

TABLE 3

Pectic substances found in the alcohol precipitate of Duncan grapefruit and Pineapple orange concentrates by colorimetric and calcium pectate methods

Fruit	Extraction method	Calcium pectate	Anhydrogalacturonic acid	Ratio: Calcium pectate ÷ anhydrogalacturonic acid
		%	%	
Duncan grapefruit	$(NaPO_3)_6$	30.4	27.8	1.09
	$(NaPO_3)_6$ and HCl	30.8	27.4	1.12
Pineapple orange	$(NaPO_3)_6$	40.8	35.4	1.15
	$(NaPO_3)_6$ and HCl	29.8	25.1	1.19
Pectic acid[b]	.....	105.8	99.7	1.06

[b]Prepared from commercial citrus pectin.

The pectic acid sample (Table 3) was prepared from commercial citrus pectin by cold saponification, precipitation with hydrochloric acid followed by a second saponification, reprecipitation with hydrochloric acid, washing free of chlorides with 70% alcohol, and oven dried. The analyses were run on a 0.1% solution by the calcium pectate and colorimetric methods.

The calcium pectate to anhydrogalacturonic acid ratio varied from 1.06 for pectic acid to 1.19 for the pectic substances found in an extract from Pineapple orange concentrate. The calcium pectate made from the pectic acid sample contained 6.1% calcium, calculated by difference from data shown in Table 3, which is probably close to a minimum calcium content for a fully-reacted calcium pectate. No exact conversion factor was found for interchanging anhydrogalacturonic acid-calcium pectate results.

DISCUSSION

All juice samples were blended in a Waring blendor to comminute the sample and to increase the rate of extraction of pectic material. The concentrates were blended before thawing to minimize enzymic action. A colloid mill with plate clearance set at 0.005 inch may be used to advantage in decreasing the difference between duplicate determinations caused by sampling errors.

To avoid filtration and the transfer of precipitates, all separations were made by centrifugation and decantation. With a centrifugal period of 10 minutes at a relative centrifugal force (R.C.F.) of approximately 1,000 times gravity, the separations were sharp and the supernatant liquid could readily be decanted.

The pectic substances were precipitated twice in boiling alcohol to remove interfering carbohydrates and at the same time concentrate the pectic material. Heating the alcoholic mixture inactivates the pectic enzymes and deaerates and flocculates the suspended pectic material so that it readily settles upon centrifugation.

Pectic substances were divided into three groups on the basis of their solubility in increasingly stronger reagents. The first 2 water extractions removed the water-soluble pectic substances of sufficiently high-methoxyl content to be termed pectins *(7)*. In citrus juices these high-methoxyl pectins give the juice its body or consistency and serve as colloidal stabilizers for the insoluble suspended solids.

Sodium hexametaphosphate soluble pectic substances are the low-methoxyl pectinates of the polyvalent cations, magnesium and calcium. The polyphosphates have a sequestering effect on the calcium and magnesium ions, thus solubilizing the low-methoxyl pectinates. The presence of low-methoxyl pectins causes the 2 defects, gelation and clarification, in frozen citrus concentrate. When present in low concentrations the low-methoxyl pectins form a flocculent precipitate with polyvalent metal ions which occludes the insoluble solids and results in clarification of the juice upon complete settling of the pectic material. At higher concentrations the low-methyl-ester pectins form a gel structure in combination with polyvalent metal ions. This gel formation in cans of frozen citrus concentrate is the defect termed "gelation" by the citrus processor.

Sodium hydroxide soluble pectic substances include the protopectin and the calcium and magnesium pectates not removed by the sodium hexametaphosphate extractions. Calcium and magnesium pectates of citrus juices are insoluble compounds formed as end products of chemical or enzymic demethylation of pectin. Protopectin is the water-insoluble parent pectic substance *(7)* which yields pectinic acids upon acid hydrolysis in citrus juices. During extended storage, hydrolysis of protopectin might increase the water-soluble pectinic acid content of a citrus juice.

The colorimetric method measures the galacturonic acid content of a sample, while the calcium pectate method measures the pectic acid content. Considering the type of determination, the colorimetric method is in good agreement with the calcium pectate method, as shown by data presented in Table 3.

The maximum absorbancy for the galacturonic acid-carbazole complex occurred at a wave length of 525 mμ. This wave length was used in determining the concentration of galacturonic acid in the described procedure because the maximum sensitivity of a colorimetric method occurs at the maximum absorbancy wave length. From the spectrophotometer curves (Figure 2) it is apparent that at a wave length of 500 mμ there is only a small amount of interference from fructose. At the 500 mμ wave length there is negligible interference from sucrose, glucose, citric acid, and ascorbic acid and this wave length might be used where increased accuracy is desired.

When sugars or polysaccharides are treated with carbazole under the same conditions of temperature and reagent concentration used above, a red color is obtained, the intensity of the color varying with the carbohydrate. The color developed by pentoses and the polysaccharides is less intense than that obtained from equal amounts of hexose sugars. The mechanism of this color reaction is not known. Furfural and hydroxymethyl furfural are formed from carbohydrates under the action of heat and acid, and these products may react with carbazole to give the characteristic red color. The red color is extremely sensitive to small amounts of water. Upon standing, the surface of the solution turns violet, due to water adsorbed from the air by the concentrated acid. The addition of small amounts of water completely decolorizes the red solution. Under sealed conditions the color gradually darkens, becoming almost black after several months.

SUMMARY

A rapid method for the estimation of pectic substances in citrus juices was developed, using the carbazole-hexuronic acid-sulfuric acid reaction. The pectic substances were divided into 3 groups on the basis of their solubility in water, sodium hexametaphosphate, and sodium hydroxide.

The procedure is suited to routine control work, with a complete analysis taking 3½ hours. Results are reported in terms of anhydrogalacturonic acid, the basic structural unit of the pectin molecule.

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THE EFFECT OF SODIUM ALGINATE ON THE DESTRUCTION OF ASCORBIC ACID[a]

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A considerable loss of vitamin C takes place during the processing and storage of food. Different ways have been found to compensate for this loss, such as adding synthetic vitamin C to the product, or using substances which have an inhibitory effect on the destruction of the vitamin. Because of the increasing interest in the use of alginates and other derivatives of alginic acid in the food industry we have investigated the effect of sodium alginate on the oxidation of vitamin C and its efficiency as an antibrowning agent. The experiments have been carried out on aqueous solutions containing various amounts of copper, and on fruit juices, varying the concentration of alginate, the pH, and the temperature. Oxygen has been supplied in some experiments by bubbling O_2 through the solutions and in other experiments by letting the solutions stand in Ehrlenmeyer flasks at room temperature.

The experimental results show that sodium alginate has a preserving effect on vitamin C.

MATERIALS

Alginate. The alginate used in our experiments ("Laminal S. F." from A/S Protan, Drammen, Norway, supplied by Kjellbergs Successors A.B., Stockholm) was a sodium alginate with a water content of 20%. Foreign substances were Na_2SO_4 (0.5%) and traces of calcium and iron. The copper content, estimated as described below, was 6.8×10^{-2} mg./g. (68 p.p.m.).

Ascorbic acid. The ascorbic acid (supplied by Merck & Co., Inc.) was assumed to be 100% pure L-ascorbic acid.

Solutions. Copper-free water was obtained by passing distilled water through the cation exchange resin Dowex 50, 60-100 mesh. Copper-free alginate solutions were prepared in the same way. Solutions containing the desired concentrations of copper were then prepared by adding anhydrous copper sulfate (Merck & Co. Inc., analytical grade).

Indophenol reagent. The 2,6-dichloroindophenol used for the determination of ascorbic acid (supplied by Hoffman-La-Roche Company) was prepared as a stock solution according to A.O.A.C. (1) and was kept at 4°C. and checked each day.

Metaphosphoric acid. The metaphosphoric acid used (supplied by Coleman and Co.) was prepared from pellets, washed with distilled water, and dried before use. The stock solution was kept at 4°C. for not longer than one week.

Distilled water. The distilled water was prepared in a tinlined still. The concentration of copper, determined as described below, was 0.15 p.p.m.

ANALYTICAL METHODS

Ascorbic acid was determined by titrating a known amount of the sample in metaphosphoric acid with 2,6-dichloroindophenol. In those cases when the total amount of vitamin C (ascorbic acid and dehydroascorbic acid) was estimated, the dehydroascorbic acid present in the sample was reduced to ascorbic acid before titration by passing H_2S into the solution for 15 min. at pH 4.5. Nitrogen was then bubbled through

[a] Patent pending.

the solution until no H_2S could be detected in the gas and the titration was carried out in the normal way.

The copper content of the water and the alginic acid was estimated colorimetrically using sodium diethyldithiocarbamate. One liter of the test solution was run through a 1.5 cm. column of the cation exchanger Dowex 50, 60-100 mesh. The copper was eluted with 3 N. HCl, 10 ml. of 0.1% sodium diethyldithiocarbamate solution was added, the mixture was diluted to 100 ml. with sodium citrate buffer, and the color developed was measured in a Beckman spectrophotometer at 450 mμ. A standard curve showing optical density versus copper concentration was plotted. It was assumed that all the copper in the test solutions was removed by ion exchange, as no copper could be detected if the analytical procedure was repeated on a solution that had passed through the exchanger. It should be mentioned that as little as 10^{-3} mg. Cu./liter of test solution could be detected.

EXPERIMENTAL

The reaction vessels were 2 gas washing bottles, one containing an aqueous solution of ascorbic acid, the other the same concentration of ascorbic acid in a solution of alginate; 60-100 mg. of ascorbic acid per 100 ml. was employed. The reaction vessels were placed in a thermostatic bath, the temperature of which could be adjusted between 20° and 50°C. ± 0.2°. The wash-bottles were connected in series and oxygen was bubbled through at a rate of 250 ml./min. as measured with a manometer (orifice type flowmeter). The oxygen was passed through a wash-bottle containing water before entering the reaction vessels in order to avoid changes in concentration and temperature in the first reaction vessel. The solutions were adjusted to the same pH, as measured with a Macbeth industrial model pH-meter. Samples were removed from the reaction vessels by means of a pipette at 10-min. intervals and immediately diluted with metaphosphoric acid and titrated as described above.

Solutions were also kept at room temperature in Ehrlenmeyer flasks. The reaction was allowed to proceed for about 120 hours and no oxygen was bubbled through. Samples were taken out at 5- to 15-hour intervals.

The ascorbic acid remaining has been calculated in percent of the original concentration. The oxidation of ascorbic acid seems to be a reaction of the first order in those cases where oxygen is supplied and the rate of destruction is not too rapid (e.g. Table 1, pH 4.5), but deviates from a first order reaction if the decay is rapid (e.g. Figure 1) or in solutions left standing in air at room temperature (e.g. Table 2). M. A. Joslyn and J. Miller *(4, 7)* have pointed out that the rate of destruction is a reaction of the first order in aqueous solutions of pure ascorbic acid and approximately of the first order in solutions containing sugars. The experimental results are shown diagrammatically or in tables.

A preliminary experiment was carried out as follows: Two solutions were prepared, one containing ascorbic acid in distilled water and the other the same amount of ascorbic acid and 0.2% alginate in distilled water. Oxygen was bubbled through at 40°C. for one hour. The results obtained are shown in Figure 1, which also shows the curves obtained with ascorbic acid solutions containing 0.2% pectin and 0.2% carboxymethylcellulose (C.M.C.). It should be pointed out that the alginate-ascorbic acid solution thus made contains twice as much copper (0.29 p.p.m.) as the aqueous solution of ascorbic acid (0.15 p.p.m.).

In order to investigate the effect of pH on the reaction, 2 series of experiments were carried out at pH 4.5 and 6.5 with solutions made as described above, one series at 30°C. with O_2 passing through the reaction vessels, the other at room temperature in Ehrlenmeyer flasks. Table 1 lists the results, and it can be seen that a better preserving effect is obtained at the higher pH.

Copper-free solutions and solutions containing 0.15, 1.2, and 10 p.p.m. of copper were prepared, the concentration of copper being the same in both the alginate solutions and the aqueous solutions of ascorbic acid. The effect of 0.1, 0.2, and 0.4% alginate on an ascorbic acid solution containing 1.2 p.p.m. of copper at pH 4 is shown in Figure 2 and in Table 2. The results plotted in the figure were obtained with O_2 bubbling through the solutions for 60 min. at 20°C. The table lists results obtained with solutions standing in Ehrlenmeyer flasks at room temperature.

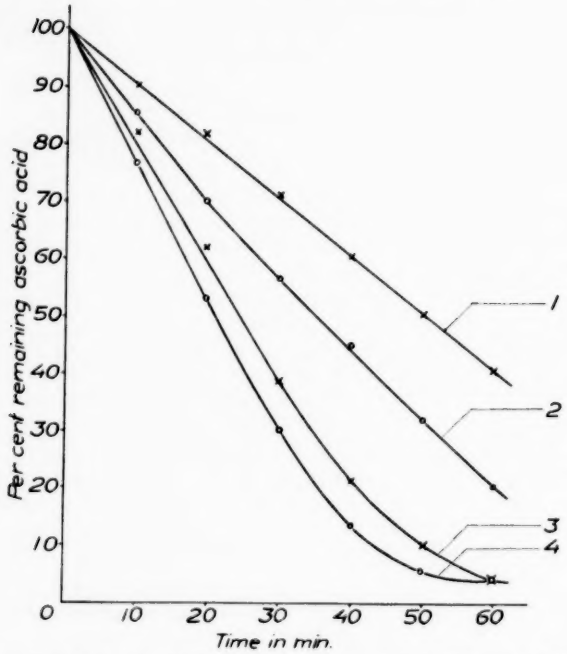

Figure 1. The effect of sodium alginate, carboxymethylcellulose and pectin on the oxidation of ascorbic acid.

Oxygen supplied at 40°C. pH = 4.

1. **0.2% sodium alginate.**
2. **aqueous solution.**
3. **0.2% carboxymethylcellulose.**
4. **0.2% pectin.**

Figure 3 shows the rate of destruction at 40°C. in 0.2% alginate solution with 0.15 p.p.m. of copper and in a copper-free medium, i.e. solutions passed through the ion exchanger. Oxygen was supplied from a cylinder, and the pH of the solutions was 4.

Table 3 gives the experimental data for 10 p.p.m. of copper at pH 4, and for 1.2 p.p.m. of copper and copper-free solutions at pH 6.

Finally Table 4 gives the results obtained with orange juice adjusted to pH 3.8 containing 0.1% alginate. Experiments were carried out with O_2 bubbling through the juice at 50°C., and with the juice in Ehrlenmeyer flasks at room temperature.

DISCUSSION

An inhibitory effect of sodium alginate on the oxidation of ascorbic acid to dehydroascorbic acid was observed even in the preliminary experiment (Figure 1). Pectin and carboxymethylcellulose increased the rate of destruction under these conditions. Pectin has been reported to accelerate the destruction of vitamin C by some authors *(8)* while others mention pectin as an inhibitor *(2)*. This difference of opinion can be due, at least partly, to the fact that different amounts of copper have been introduced into the ascorbic acid solutions by the pectins used. W. Grab *(2)* has

TABLE 1

The effect of sodium alginate on the destruction of ascorbic acid at different pH

pH 4.5					
30°C., O_2 supplied			Room temp. in air		
Time, min.	Percent rem. asc. acid		Time, hours	Percent rem. asc. acid	
	No algin.	0.2% algin.		No algin.	0.2% algin.
0	100	100	0	100	100
10	91.3	100	6	89.5	94.5
20	81.5	97.9	16.5	76.8	86.2
30	73.8	94.8	20.5	72.6	80.7
40	68.9	90.8	32.5	57.8	73.1
50	63.1	88.7	44.0	47.4	67.8
60	55.4	84.6	55.0	35.8	63.5
....	....	....	67.0	31.6	60.2
....	....	....	74.0	25.3	58.1
....	....	....	92.0	16.8	53.7
....	....	....	98.0	15.2	52.6
....	....	....	114.0	11.8	50.5
pH 6.5					
0	100	100	0	100	100
10	77.4	94.8	6	82.0	87.6
20	52.7	87.7	16.5	62.9	82.1
30	35.5	79.3	20.5	56.2	80.8
40	26.9	69.4	32.5	36.0	79.7
50	20.4	61.8	44.0	22.5	78.6
60	14.0	53.7	55.0	11.2	76.5
....	....	....	67.0	4.5	74.2
....	....	....	74.0	3.6	71.8
....	....	....	92.0	1.6	65.2
....	....	....	98.0	1.5	64.2
....	....	....	114.0	1.5	60.7

TABLE 2

The effect of different concentrations of alginate on the destruction of ascorbic acid

Time, hours	Room temp., pH 4.0, 1.2 p.p.m. of Cu. Percent rem. ascorbic acid			
	No algin.	0.1% algin.	0.2% algin.	0.4% algin.
0	100	100	100	100
4.0	79.8	84.7	88.4	93.8
7.5	67.0	80.6	83.0	91.3
13.0	53.2	68.3	74.5	81.4
24.0	39.1	58.1	59.7	69.4
50.0	13.3	23.7	30.8	38.4
72.0	4.9	7.1	13.5	17.8
	tot. vit. C	tot. vit. C	tot. vit. C	tot. vit. C
120.0	27.7	27.6	36.1	45.6

recently pointed out that concentrations of copper as low as 0.001 p.p.m. are enough to cause a noticeable increase in the rate of oxidation of ascorbic acid. Even in our experiments, a higher concentration of copper in the solutions containing pectin or carboxymethylcellulose than in the aqueous solution of ascorbic acid is probably responsible for the more rapid

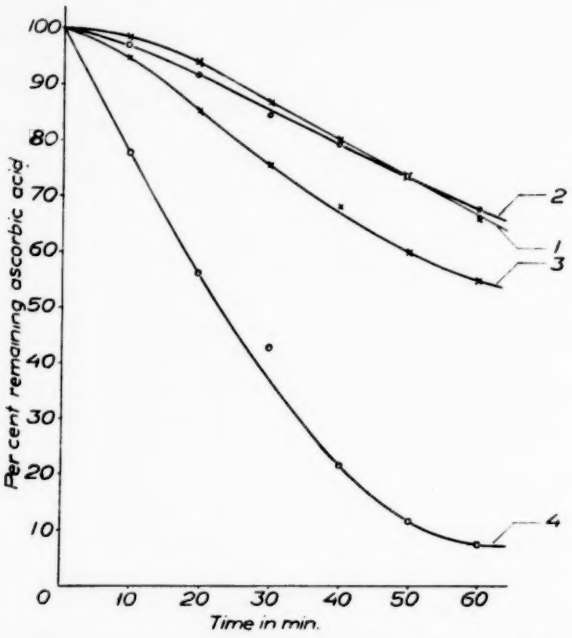

Figure 2. The effect of different concentrations of sodium alginate on the destruction of ascorbic acid.

Oxygen supplied at room temperature (20°C). pH = 4. 1.2 p.p.m. copper.

1. **0.4% sodium alginate.**
2. **0.2% sodium alginate.**
3. **0.1% sodium alginate.**
4. **aqueous solution.**

TABLE 3

The effect of sodium alginate on the destruction of ascorbic acid, varying the concentration of copper and the pH

20°C., O_2 supplied			20°C., O_2 supplied			40°C., O_2 supplied		
pH 4.0, 10 p.p.m. Cu.			pH 6.0, 1.2 p.p.m. Cu.			pH 6.0, Cu.-free		
Time, min.	Percent rem. ascorbic acid		Time, min.	Percent rem. ascorbic acid		Time, min.	Percent rem. ascorbic acid	
	No algin.	0.2% algin.		No algin.	0.2% algin.		No algin.	0.2% algin.
0	100	100	0	100	100	0	100	100
10	79.7	87.8	10	50.6	54.3	10	99.5	99.3
20	58.1	73.3	20	17.5	19.6	20	94.5	93.2
30	45.9	58.8	30	7.4	8.7	30	87.1	88.0
40	36.4	47.7	40	4.8	5.4	40	81.7	82.3
50	31.0	38.8	50	4.0	2.6	50	76.6	78.9
60	27.0	31.1	60	3.1	1.7	60	71.2	73.4
70	22.9	24.4	70	....	....	70	62.1	64.8

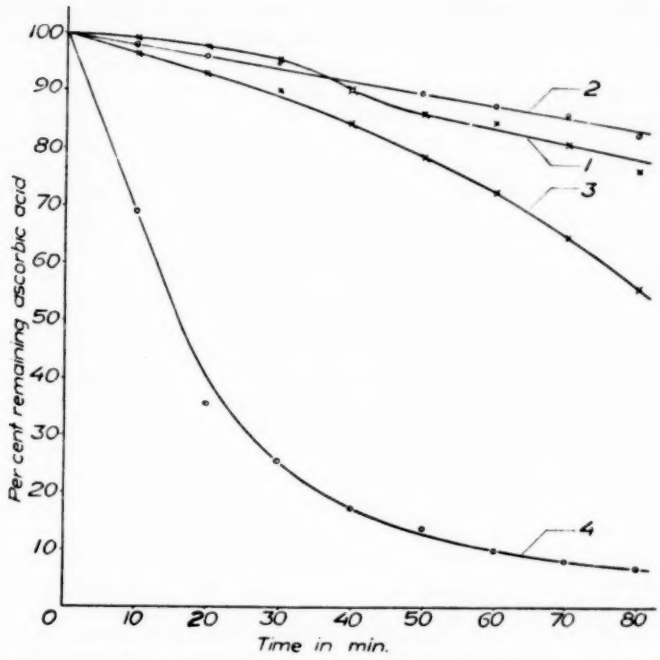

Figure 3. The effect of sodium alginate on the destruction of ascorbic acid in solutions containing different amounts of copper.

Oxygen supplied at 40°C. pH = 4.

1. **Copper free solution, 0.2% sodium alginate.**
2. **Copper free solution, no sodium alginate.**
3. **0.15 p.p.m. copper, 0.2% sodium alginate.**
4. **0.15 p.p.m. copper, no sodium alginate.**

TABLE 4

The effect of alginate on the destruction of ascorbic acid in orange juice at pH 3.8

Temp. 50°C., O_2 supplied			Room temp., in air		
Time, min.	Per cent rem. asc. acid		Time, hours	Per cent rem. asc. acid	
	No alginate	0.1% alginate		No alginate	0.1% alginate
0	100	100	0	100	100
10	98.0	98.2	17	88.1	93.1
20	89.9	89.5	25	79.7	84.0
30	81.0	80.4	42	69.3	80.5
40	68.2	70.8	48	63.5	74.3
50	53.5	60.2	67	45.9	65.6
60	36.9	47.4	91	21.7	46.5
....	....	....	97	16.1	43.1
....	....	....	117	2.8	28.0

destruction of the ascorbic acid in the presence of these two substances. The alginate can apparently exert a preserving effect in spite of the higher concentration of copper in the alginate-ascorbic acid solution.

The experiments summarized in Figure 3 and Table 3 show that alginate exerts its preserving effect only if copper ions are present in the ascorbic acid solution. It has already been mentioned (Table 1) that the alginate had a greater preserving effect at pH 6.5 than at pH 4.5 if the copper content of the solutions was relatively low (0.29 p.p.m.). When the concentration of copper was higher the inhibitory effect of the alginate was superior at the lower pH (Table 3). These experiments have been repeated several times under different conditions, with the same results. We can offer no plausible explanation for this phenomenon.

Our experiments thus provide evidence of the inhibitory effect of sodium alginate upon the oxidation of ascorbic acid to dehydroascorbic acid. This reaction is known to be influenced by pH, temperature, oxygen, and the presence of enzymes and metal ions, especially copper. The further conversion of dehydroascorbic acid to 2,3-diketo-L-gulonic acid is not an oxidation, and copper is without effect upon this reaction (3). We have not investigated this step, which leads from the two active forms of vitamin C—ascorbic acid and dehydroascorbic acid—to the biologically inactive gulonic acid in detail. Table 2 shows an example of how total amount of vitamin C is influenced by the presence of various amounts of sodium alginate, and it seems likely that alginate can have some inhibitory effect upon the conversion of dehydroascorbic acid to 2,3-diketo-L-gulonic acid. M. A. Joslyn and Helen Supplee *(5)* have found that sugars can inhibit this reaction and that the extent of inhibition increases with increasing concentration of the sugar.

Some experiments designed to test the efficiency of alginates as antibrowning agents have been made. They were undertaken because ascorbic acid has been reported to decrease the rate of browning of apples *(9)* and alginates to improve the structure of frozen strawberries *(10)*. Experiments have been carried out in two different ways, *viz.*, by measuring the degree of browning of apple juice, and by observing the effect of alginate on quick-frozen slices of apples. Oxygen was bubbled through apple juice adjusted to pH 3.8 to prevent the precipitation of alginic acid, samples were removed at intervals and the browning of the juice with or without sodium alginate was measured with a Beckman spectrophotometer. The slices of apples were frozen in mixtures of sugar containing a few percent of alginate, stored at −20° C. for 2 months and thawed and the degree of browning and the structure was observed. We could find no significant decrease in the rate of browning when sodium alginate was added. As a mixture of pectin and ascorbic acid seems to be a more effective antibrowning agent than ascorbic acid alone *(6)*, it is not improbable that the same is true for mixtures of alginate and ascorbic acid.

SUMMARY

The influence of sodium alginate on the oxidation of ascorbic acid to dehydroascorbic acid has been investigated, varying the concentration of alginate, the temperature, the pH, and the concentration of copper in the

test solutions. It has been found that sodium alginate can inhibit the oxidation in ascorbic acid solutions containing copper ions. Sodium alginate was also tested as a possible antibrowning agent, but appeared to be without effect.

Acknowledgment

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ANTIBACTERIAL ACTIVITY OF EDIBLE PLANT EXTRACTS[a,b]

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In the development of adequate heat processes for canned foods, it has been noted that a food spoilage organism may have different heat resistances in various food products. The pH values of the products do not offer in themselves an adequate explanation for the variable heat resistance of the organism. Sognefest and associates *(8)* noted that the F value obtained for the spores of test organisms varied with the type of product in which the spores were heated and incubated as well as the pH of the media. It was also demonstrated by Reed and associates *(5)* that some semi-acid products, such as green beans and sweet potatoes, exhibited an inhibitory effect upon the growth of the most heat-resistant surviving spores of Putrefactive Anaerobe No. 3679. Reynolds and co-workers *(6)* also described the inhibition of growth of heat-resistant spores of Putrefactive Anaerobe No. 3679 and found it to be exhibited by many low-acid vegetables. This would suggest that some substances are present in edible plants which have antibacterial activity.

The widespread antibiotic activity of plants is attested by the published reports of various investigators. Osborn *(4)* tested the antibiotic activity of water extracts from 2,300 species of plants and found varying degrees of bacterial inhibition against test organisms. Fontaine *(2)*, Bruckner *(1)*, and Scott *(7)*, and their associates, described the isolation and partial purification of antibiotic agents derived from tomato leaves, sweet potatoes, and banana skins. Spoehr and co-workers *(9)* noted the antibacterial action of fatty acids obtained from cabbage, turnips, carrots, and other edible plants. L. B. Jensen and his associates *(3)* have utilized various plant extracts in meat pickling solutions yielding a product exhibiting marked increased resistance to the development of pathogenic and food poisoning microorganisms.

The purpose of this investigation was to study the antibacterial activity of particular edible plant extracts and the ability of these extracts to reduce the heat tolerance of food spoilage organisms.

EXPERIENTAL PROCEDURE AND RESULTS

Preparation of extracts. Two pounds of the leaves, stems, and edible portion of the particular plant under investigation were obtained fresh from the field, washed thoroughly, drained, and chopped finely.

One pound of the mixed, chopped plant material was macerated into a fine slurry with the addition of 200 ml. of sterile distilled water, centrifuged and sterilized by filtering

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[b] Presented at the Twelfth Annual Meeting of the IFT, Grand Rapids, Michigan, June 11, 1952.

the supernatant through a Seitz filter. A 100-ml. portion of the sterile filtrate was concentrated by lyophilization, and the final concentrate was stored at 2-4°C.(35.6-39.2°F.).

The remaining one pound of plant material was treated as above with the exception that 95% ethanol was used in extraction and concentrated to dryness by means of vacuum distillation.

Method of assay. The organisms employed for screening purposes in this investigation were *Escherichia coli* A.T.C.C. 26, *Micrococcus pyogenes* var. *aureus* (FDA 209 P), *Saccharomyces ellipsoideus* var. *tokay*, and *Bacillus subtilis*, obtained from stock cultures. Sterile 90-mm. petri dishes, containing 20 ml. of solidified agar media (0.5% tryptone, 0.5% proteose peptone, 0.5% yeast extract, 0.5% dextrose) were warmed and overlayed with 4 ml. of the base agar which had been seeded with the test organism.

To test the antibacterial activity of the dehydrated water extracts, the powdered material from 100 ml. of extract was dissolved in 10 ml. of sterile phosphate buffer, pH 7.0, 0.066 M., Sterile No. 2 cotton dental rolls, one cm. in length, saturated with 1.5 ml. of these rehydrated concentrates, were placed on the solidified seeded agar plates. The alcoholic extracts were assayed as above. Plates were incubated for 24 hours at the temperature optimum for the growth of each test organism. Antimicrobiological activity was determined by relative size of zones of inhibition.

In this manner 30 edible plants were screened for antibacterial substances. These were as follows: asparagus, beets (sugar), beets (red), broccoli, cabbage, carrots, celery, chard, chicory, Chinese cabbage, cucumber, dill, egg plant, green beans, green pepper, kale, muskmelon, okra, onion (Bermuda), parsnips, pumpkin, rhubarb, rutabaga, spinach, squash (Summer), squash (hybrid), sweet corn, sweet potato, turnips, and tomatoes. The results (Table 1) indicate that many of the plant extracts contained inhibitory substances. Those extracts possessing activity against routine screening organisms were also studied for their ability to reduce the heat tolerance of resistant bacterial spores.

Preparation of spore suspension of Flat Sour (FS) No. 787. A spore suspension of Flat Sour No. 787 was obtained by growing the organism[c] for 48 hrs. at 55°C.(131°F.) on the surface of nutrient agar in Roux flasks, and aseptically harvested by washing the surface with 100 ml. of sterile phosphate buffer pH 7.0, 0.066 M. The washings were filtered, boiled for one minute at 100°C., and stored at 2-4°C.(35.6-39.2°F.).

Spore destruction rate tests. The determination of spore destruction rates was made in a 1,000-ml. 3-necked distilling flask. A glass stirring rod, enclosed in an 8-inch close-fitting, glass tube held in a rubber stopper in the center hole, was employed to vigorously agitate the spore suspension. A thermometer mounted in a rubber stopper was placed in one of the side holes. An Escher stopper was placed in the remaining hole so that a sample of the heated spore suspension could be withdrawn aseptically at varying time intervals.

The contents were held at a temperature of 201°F.(93.8°C.) ± 0.5°F.(0.27°C.) in a water bath. Two minutes were required to reach this operating temperature.

The lyophilized material from 100 ml. of plant extract was dissolved in 50 ml. of neutral phosphate buffer, and inoculated with one ml. of the spore suspension. After thorough mixing, a one-ml. aliquot was immediately removed to ascertain the number of viable spores present before heat treatment by dilution plating techniques. This suspension was then placed in the distilling flask heating chamber, heated, and spore destruction rates were determined by plating serial dilutions of one-ml. aliquots of the sample taken at various time intervals.

To determine the initial effect of the extract on the viability of the spores, 50 ml. of phosphate buffer pH 7.0, 0.066 M., was inoculated with one ml. of the spore suspension and plated in a like manner. Initial average counts of both the extracts and the buffer suspensions are presented in Table 1. Except for lupulon, counts were essentially in agreement.

The extracts employed in this series of tests were bean, carrot, tomato plant, and diluted crystalline lupulon.[d] An additional treatment of 174 p.p.m. of indol 3 acetic

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[c] The original culture was obtained from the Continental Can Company Research Laboratories, Chicago, Illinois.

[d] Obtained from the Western Regional Research Laboratory, BAIC, USDA, Albany, California.

TABLE 1

Effect of 12 edible plant extracts against four test organisms

Plants	Organism: Zones of inhibition in millimeters			
	E. coli	*M. P. aureus*	*B. subtilis*	*S. ellipsoideus*
Carrot	20(A)	22(A)	24(A)	.....
Green Beans	18(A)	22(A)	24(A)	.....
Onion (Bermuda)	20(A)	15(A)	17(A)	19(A)
Tomato	.....	15(W)	17(W)	15(W), 18(A)
Celery	.....	15(A)	17(A)	.....
Chicory	.....	.....	16(A)	.....
Cucumber	.....	.....	15(W)	.....
Chard	.....	.....	.....	19(W)
Okra	.....	14(A)	.....	.....
Rhubarb	16(A)	22(A)	17(A)	.....
Sweet Corn	.....	.....	14(A)	.....
Turnips	.....	.....	13(A)	.....

A = Alcohol extract.
W = Water.

acid, an auxin which has been shown to stimulate plant growth in minute amounts, in neutral phosphate buffer, was also included. These results are shown in Tables 2 and 3, and graphically in Figure 1.

DISCUSSION

The results (Table 1) indicate that the crude extracts of many edible plants contain substances which will inhibit the growth of many micro-organisms. The role these substances play in the control of spoilage cannot be stated, but the fact remains that their presence does exert antibacterial influence. The presence of these substances in contact with the spores of a food spoilage organism, such as Flat Sour No. 787, in conjunction with a mild heat treatment shows a striking sporicidal effect. Reduction in the numbers of surviving spores in the presence of these substances may also account for the differences in the F values obtained when employing purees

TABLE 2

Rate of destruction on spores of Flat Sour No. 787 in phosphate buffer heated at 201°F.(93.8°C.)

Time heated in minutes	Temperature	Number of surviving organisms /ml.
	°F.	
0	75	15,700
½	165	15,600
1	185	14,500
2	201	14,100
4	201	14,000
6	201	11,000
8	201	9,000
10	201	7,500
15	201	4,000
20	201	980
29	201	225
32	201	150
35	201	0

TABLE 3

Effect of several plant extracts on survival of spores of Flat Sour No. 787 heated at 201°F.(93.8°C.)

Time heated in minutes	Temperature	Number of surviving organisms/ml.				
		Tomato plant	Green beans	Carrot	Lupulon	Indol 3 acetic acid
	°F.					
0	75	35,000	34,000	42,000	3,200*	24,000
1	185	500	9,000	1,000	100	.....
2	201	.....	100	.....	.....	.....
3	201	65	0	200	0	5,000
5	201	0	.....	0	.....	.....
6	201	.....	.....	.....	.....	300
9	201	.....	.....	.....	.....	65
15	201	.....	.....	.....	.....	10
18	201	.....	.....	.....	.....	0

*Phosphate buffer control of same spore inoculum contained 30,000 spores/ml. Concentration of Lupulon used was 100 p.p.m.

to ascertain adequate heat processes for canned foods. Studies are underway in these laboratories with putrefactive anaerobes. Preliminary results are encouraging.

If substances normally present in foods are found to be effective in a synergistic manner with mild heat treatments against the spores of food spoilage organisms, it would be desirable to approach the problem of reducing the heat resistance of bothersome thermophiles and mesophiles using naturally occurring materials. The use of edible plant extracts may not pose the serious problem encountered with the use of other antibiotics such as subtilin and penicillin. Another factor to be considered is the

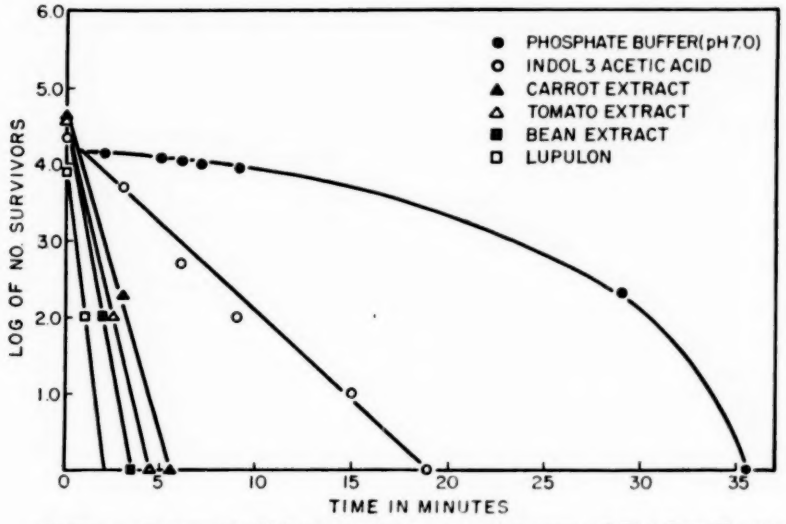

Figure 1. Influence of edible plant extracts on survivor curve of Flat Sour No. 787 heated at 201°F.

effect of these substances when present in the hydrolysate of plants being assayed microbiologically for vitamins and amino acids. Preliminary tests show that high dilutions of the extracts possess a bactericidal potency against *Leuconostoc mesenteroides* and *Lactobacillus arabinosus*.

Identification of these inhibitory substances is not yet complete. It is quite probable that the component responsible for the antimicrobial activity in tomato extract was the glycosidal alkaloid crystallized by Fontaine and co-workers *(2)*. Some of the antibacterial activity obtained from these extracts may also be due to fatty acids present in the alcoholic fractions.

Lupulon and the auxin indol 3 acetic acid in 100 and 174 p.p.m., respectively, were effective in reducing the heat tolerance of Flat Sour No. 787 spores.

SUMMARY

Many edible plant extracts were found to contain antibacterial substances of varying degrees of activity. Several of these extracts were employed in conjunction with mild heat treatments in spore destruction studies of Flat Sour No. 787.

Preliminary results indicate that these substances may be used as a means of reducing the heat tolerance of spores of food spoilage organism.

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THE INFLUENCE OF A PREHARVEST FOLIAR SPRAY OF MALEIC HYDRAZIDE ON THE SPECIFIC GRAVITY OF POTATOES AND THE QUALITY OF POTATO CHIPS[a]

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Possible improvement in the quality of potato chips by spraying the vines with maleic hydrazide has been suggested by Paterson, Wittwer, Weller, and Sell *(8)*. Denny and Thornton *(5)* have shown that reducing sugars are largely responsible for the dark color formed in frying. Treatment with maleic hydrazide *(8)* appeared to result in lower contents of reducing sugars, while at the same time reducing the tendency to sprout in storage. Thus, there is indication that treatment with maleic hydrazide might be beneficial in at least two ways, i.e. improvement in color of potato chips and decrease in sprouting and shrinkage in storage. In 1950 and 1951, potato vines were given a preharvest foliar spray with maleic hydrazide (2,500 p.p.m.) and the effects on specific gravity of the tubers and the quality of chips are herein reported.

EXPERIMENTAL METHODS AND PRELIMINARY OBSERVATIONS

Irish Cobbler potatoes were planted on May 19, 1950, on a productive Hillsdale sandy loam soil. Rows were 36 inches apart and seed pieces placed at 12 inch intervals. Approximately 7 weeks before harvest (July 15) the vines were sprayed with the equivalent of 150 gallons per acre of a 2,500 p.p.m. solution of maleic hydrazide. The method of spray application and experimental design was that described by Paterson, *et al. (8)*. Potatoes harvested from vines receiving no chemical treatment were used as a control comparison. Immediately following harvest on August 25, random samples consisting of 10 tubers each were taken from treated and non-treated plots and held in storage at temperatures of 45 ± 2°F.(7°C.) and 55 ± 2°F.(13°C.).

On March 29, 1951, after 7 months, the potatoes were removed from storage and "conditioned" for 20 days at room temperature of 73 ± 3°F.(23°C.). Specific gravity determinations were then made according to the brine flotation method described by Clark, Lombard, and Whiteman *(4)*. Tubers from vines sprayed with maleic hydrazide appeared to be slightly higher in specific gravity than those from the control plants. Repetition of the tests the following year on the 1951 crop with larger samples and with additional varieties justified this conclusion.

Following specific gravity determinations the tubers were peeled and uniformly sliced. The slices were separated, washed under running tap water to remove adhering starch and to separate the slices and then fried in "Primex"[b] at an initial temperature of 385°F.(196°C.) and final removal temperature of 348 ± 2°F.(176°C.). For identification, holes were punched in slices prepared from potatoes harvested from plants sprayed with maleic hydrazide or those of the same treatment were threaded together. Chips processed from tubers harvested from plants sprayed with maleic hydrazide were definitely lighter in color and judged more acceptable than chips made from non-treated potatoes.

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[a] Journal Article No. 1397 of the Michigan Agricultural Experiment Station.

[b] Procter and Gamble, Cincinnati, Ohio.

Detailed Studies on 1951 Crop

Russet Rural, Irish Cobbler, and Sebago potatoes—standard varieties for chipping in Michigan—were planted May 21 in a randomized block consisting of duplicate plots of 9 x 96 feet for each variety. Method of planting and soil type were similar to those of the previous year's study. Supplementary irrigation was employed as needed and standard disease and insect control measures utilized. Half of the vines of each duplicate plot of each variety were sprayed on July 20 with 2,500 p.p.m. of maleic hydrazide and half were left as controls without treatment. Triton B1956[c] at 0.1% was used as a wetting agent.

Specific gravity and chip color before storage of tubers. The potatoes were harvested September 21 and held in the dark in an open shed until October 14, following which a random sample of 60-80 U. S. No. 1 tubers from each replicate of each treatment of the 3 varieties was then taken for specific gravity determinations. It is apparent from Table 1, that the tubers of all varieties which received maleic hydrazide averaged higher specific gravities than the controls.

Two tubers each from non-treated and treated vines were selected in the specific gravity groups "under 1,063," from "1.063-1.070," and "over 1.080" to evaluate the effect of chemical treatment on chip color. The tubers were uniformly sliced from the basal to apical ends, washed, and fried in the manner previously described. Each chip was then scored according to an arbitrary color spectrum.[d] Comparative chip color ratings are listed in Table 2. The data suggest that within the same specific gravity groups, tubers harvested from vines sprayed with maleic hydrazide produced the most desirable light brown colored chips; however, the maleic hydrazide treatment did not improve the "under 1.063" or "1.063-1.070" potatoes sufficiently to place them in the acceptable class.

[c] Rohm and Haas, Philadelphia, Pennsylvania.

[d] Designed by staff members, Department of Farm Crops, Michigan State College.

TABLE 1

Effect of a preharvest foliar spray of maleic hydrazide (2500 p.p.m.) on the specific gravity of Russet Rural, Irish Cobbler and Sebago potatoes following harvest (October 15, 1951)

Variety	Number of Tubers in Specific Gravity Groups				Total readings
	Under 1.063	1.063 to 1.070	1.070 to 1.080	Over 1.080	
Russet Rural					
Control	7	9	32	28	76
Maleic hydrazide	0	4	37	29	70
Irish Cobbler					
Control	8	9	30	21	68
Maleic hydrazide	0	3	30	30	63
Sebago					
Control	6	14	29	12	61
Maleic hydrazide	5	4	37	16	62

TABLE 2

The effect of a preharvest foliage spray of maleic hydrazide on the chip color ratings of Sebago potatoes of the same specific gravity following harvest (October 18, 1951)

Treatment	Average Ratings[e] of 10 Chips in Each Specific Gravity Group		
	Under 1.063	1.063-1.070	Over 1.080
Control	5.7	7.1	8.1
Maleic hydrazide	6.8	7.3	8.3

[e] 1=extremely dark; 10=light yellow; 8.5-9=golden yellow (most desirable); 7.5-10=acceptable.

Chip color after cold storage of tubers. After storage at 37 ± 1°F.(3°C.) for 4½ months (on February 28, 1952), slices from each tuber of each specific gravity group from maleic hydrazide treated and control lots of the 3 varieties were immediately processed and given chip color ratings. Potatoes were sliced in the usual longitudinal manner, and the sixth to eighth slice from the outside retained for chipping. The remaining tuber portions were then held at a room temperature of 78 ± 2°F.(26°C.) for 43 days to provide the conditioning essential for possible development of desirable chip color in tubers of low (below 1.063 and 1.063-1.070) specific gravity *(6, 11, 12, 15)*. Following the 43 days' exposure at room temperature, the formerly sliced tubers representing the various varieties, treatments, and specific gravity groupings were again sampled and fried together in the usual manner on April 12, 1952. Following processing, each chip was given a color rating. The comparable chip ratings and specific gravity groupings for Russet Rural, Irish Cobbler, and Sebago, following the various conditions of storage and treatment, are given in Table 3. The number of tubers sampled after conditioning was less than before since breakdown and decay had occurred in some of the tuber pieces subsequent to the first sampling. Potatoes harvested from vines treated with maleic hydrazide showed a tendency towards a higher specific gravity and yielded chips of superior color within the same specific gravity groups.

The data (Table 3) emphasize that the effects of storage temperatures and variety on chip color were of great importance and more pronounced than those from maleic hydrazide. High specific gravity tubers produced chips of better color than did those of low specific gravity.

No objectionable effects in color, flavor, or texture of chips were observed as a result of spraying the potato vines with maleic hydrazide. There was a clear-cut trend toward tubers of higher average specific gravity from the treated vines. Although the improvement in color was slight as a result of spraying with maleic hydrazide, it was clearly perceptible in all specific gravity groups at harvest time, directly out of cold storage, and after reconditioning. Thus, in addition to the apparent advantages resulting from the inhibition of sprouting, field spraying with maleic hydrazide appeared to have beneficial effects on the quality of tubers produced.

DISCUSSION

The results herein show that a preharvest foliage spray of 2,500 p.p.m. of maleic hydrazide resulted in an increase in the specific gravity of the tubers and improved the color of chips which were processed from them. Clark, Lombard, and Whiteman *(4)* have reported that high specific gravity tubers have higher cooking quality and Wright and Whiteman *(16)* have suggested that chips of best appearance and quality are processed from tubers of relatively high specific gravity.

Maleic hydrazide, originally reported as a unique growth regulant *(10)* and later as a sprout inhibitor for potatoes *(7, 8)* and other crops *(13, 14)*, may reduce the activity of the phosphorylase system which normally controls the degradation of starch to sugar in potato tubers held at relatively low storage temperatures *(1)*. The tendency to sprout and the sugar content of potatoes are inversely related *(3)* while the sugar content gradually increases in potatoes transferred to low (1°C. or 34°F.) and decreases with exposure to high (15°C. or 59°F.) temperatures *(2)*. During the conditioning of the tubers at room temperature, it was observed that the sprouting tendency among control lots was greatest in the low specific gravity tubers. The general improvement in the color of chips of potatoes treated with maleic hydrazide appears to be related to slight increases in specific gravity, and to a decrease in reducing-sugar content *(8)*. It should be emphasized, however, that potatoes treated with maleic hydrazide still needed a holding period of high temperature after cold storage to give

TABLE 3

Effects of a preharvest foliage spray of maleic hydrazide (2500 p.p.m.) on chip color ratings and specific gravity of Russet Rural, Irish Cobbler and Sebago potatoes after 4½ months storage at 37°F. and following 43 days of conditioning at 78 ± 2°F.

Variety	After Cold Storage (February 28, 1952)						After Conditioning (April 12, 1952)					
	Control			Maleic hydrazide			Control			Maleic hydrazide		
	Under 1.063	1.063 to 1.070	Over 1.080	Under 1.063	1.063 to 1.070	Over 1.080	Under 1.063	1.063 to 1.070	Over 1.080	Under 1.063	1.063 to 1.070	Over 1.080
Russet Rural												
Number of tubers	6	8	13	0	4	14	0	6	10	0	4	11
Percent of total	22	30	48	....	22	78	....	38	62	....	27	73
Average chip ratings[1]	1.5	1.8	4.1	....	3.1	5.3	....	7.7	7.8	....	8.8	9.4
Irish Cobbler												
Number of tubers	5	8	13	0	3	13	2	7	9	0	3	11
Percent of total	19	31	50	....	19	81	11	39	50	....	21	79
Average chip ratings	1.0	1.3	2.7	....	1.3	3.1	2.0	6.6	7.0	....	7.0	7.8
Sebago												
Number of tubers	4	10	7	3	2	9	4	8	2	3	2	9
Percent of total	19	48	33	21	14	65	29	57	14	21	14	65
Average chip ratings	2.2	2.9	5.0	2.3	3.5	5.2	4.0	6.3	8.0	4.7	6.9	8.4
Total number of tubers	15	26	33	3	9	36	6	21	21	3	9	31
Percent of total	20	35	45	5	20	80	12	44	44	7	21	72
Average of all chip ratings	1.5	2.0	3.7	2.3	2.6	4.5	3.3	6.8	7.5	4.7	7.8	8.5

[1] 1=extremely dark; 10=light yellow; 8.5-9=golden yellow (most desirable); 7.5-10=acceptable.

chips of an acceptable color. Neither did maleic hydrazide prevent a lowering in the chip color ratings of tubers processed following the low temperature storage period. It may be that maleic hydrazide treated tubers can be "conditioned" for chipping in shorter periods and at lower temperatures because of an initially lower reducing sugar content. It is reasonable to suggest, additionally, that tubers receiving a maleic hydrazide treatment would give a better yield of chips, since sprouting and respiration during storage and conditioning are greatly decreased *(9)*.

Various anatomical aspects were associated with the brown coloration pattern of potato chips. It was observed in chips of tubers harvested from maleic hydrazide treated vines that the narrow band of cortex storage tissue immediately within the periderm was invariably lighter in color than that of the controls. However, in the controls, the vascular ring was much more darkened than in chips processed from tubers of maleic hydrazide treated vines. It was further observed within the main storage tissue of the tuber, regardless of vine treatment, that the browning of the chips was most prominent at the basal end.

SUMMARY

Maleic hydrazide applied as a preharvest foliage spray (2,500 p.p.m.) to Russet Rural, Irish Cobbler, and Sebago potatoes resulted in a larger number of tubers in higher specific gravity groupings than was found in comparable tubers harvested from non-treated plots. Within the same specific gravity groupings, following harvest, maleic hydrazide treated Sebago potatoes gave chips which were slightly superior to the controls in color quality.

Subsequent to 5 months' storage at 37°F.(3°C.) and again following 43 days of conditioning at 78°F.(26°C.), Russet Rural, Irish Cobbler, and Sebago potatoes harvested from vines sprayed with maleic hydrazide and of given specific gravity were superior according to arbitrary chip color ratings as compared with controls.

Some biochemical and anatomical aspects of the effects of maleic hydrazide on chipping quality of potatoes are discussed.

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COLLOIDAL CONSTITUENTS OF DARK BUCKWHEAT HONEY

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In spite of the extensive research on the chemistry of honey during the past hundred years, knowledge of the properties of the proteinous and pigmenting constituents is far from complete. Results, however, of recent research—utilizing ultrafiltration and spectrophotometry—have furnished interesting data *(5)*.

As to the origins of the minor constituents of buckwheat honey, it may be that they can be ascribed to the nectar, to natural impurities such as propolis, or to the products of chemical changes during maturing and storage.

Concerning the color of honey many theories have been offered, but none has as yet proved quite satisfactory. Darkening can be caused by the maltreatment of honey, such as overheating or alkalinization (which can be disregarded in the present study). Climatic, seasonal, geographical, and biological factors have been observed to influence the color of honey. Mineral content, especially manganese and copper *(7)*, have also been blamed. It has been maintained that the color derives from dyes *(1, 6, 8)* but this would not explain differences in the hue and grade of saturation. Although further listing of theories and their shortcomings could be made, it is obvious that opportunity exists for much further research in the field of the minor constituents of honey.

Inasmuch as there appear to be no data in the literature on the molecular weight of the colloidal components of honey it was considered that here would be a promising source for obtaining useful information on honey. Results of this study are reported in succeeding pages.

Preliminary considerations. For technical reasons it was found convenient to deal with dyes of lower molecular weight apart from those constituents having molecular weight over 5,000. Coloring compounds such as carotenes, chlorophylls, and anthocyanines can be extracted from honey with organic solvents if the honey is dehydrated with hygroscopic materials, such as anhydrous calcium sulphate. This extract will readily subdivide into several components if run through a chromatographic column of aluminum oxide. Some of the separated dyes can then be identified with ultraviolet light or spectrophotometry. The results of a study of these coloring constituents of lower molecular weight are reported elsewhere *(3)*.

The minor constituents of honey having molecular weights higher than 5,000 can be found in the form of colloids. They are probably proteins, peptides, or polysaccharides. It has to be kept in mind, however, that proteins will combine with non-proteinous substances to form conjugated proteins or symplexes or cenapses. Some of these compounds exist as such in organisms; others are formed only during their isolation. A few of these protein-nonprotein compounds are specific and can be used for the

determination of the protein in question. It is remarkable that methyl orange will combine readily with serum albumin, only to a very limited extent with lactoglobulin, and not at all with insulin, pepsin, or trypsin. Proteins also combine with carotenes to form colored lipoproteins *(2)*. These have been investigated only in certain animal organs. The fact that proteins combine frequently with free amino acids and their derivatives may justify the speculation that the formation of melanoidins in honey is partially due to a combination of tyrosine with proteins, since tyrosine is easily oxidized by enzymes to dark-colored indoles *(4, 9)*. This should not exclude the development of dark coloring compounds, during the storage of honey, through Maillard's aldol condensation or other browning reactions.

METHODS AND MATERIALS

The molecular weight of the minor constituents of honey, if higher than 5,000, can be determined by various means—osmometry, light-scattering, and sedimentation rate as obtained in the ultracentrifuge. Sedimentation rate, however, is not of itself an exact indication of molecular weight since the density, size, and shape of colloids have an influence on the gradient figure obtained. It is possible, however, to calculate the influence of several of these factors with good approximation. In some cases the sedimentation rate is related to the frictional coefficient. Within certain limits, molecular weight can be computed from this, but it must be assumed that the particles are sphere-shaped—which would not be correct for many proteins—and that the specific volume of the particles can be estimated to be 0.75—which bears a possible error of 5%. The application of this method is therefore restricted.

To permit the application of the gas constant RT, infinite dilution is called for. With this understanding the molecular weight M can be derived by the Svedberg formula

$$M = \frac{RTs}{D(1-Vp)}$$

for the dynamic state; and if equilibrium is reached

$$M = \frac{2RT \log e_2/e_1}{(1-V)(x_2^2-x_1^2)w^2}$$

where

R = gas constant (8.13×10^7)
T = absolute temperature
s = sedimentation rate
D = diffusion constant
Vp = partial specific volume of solute (0.75)
w = angular velocity of rotor
e_1 and e_2 concentrations at radial distances x_1 and x_2.

The sedimentation rate will give also the size of the particles, provided the particles are sphere shaped and in extremely diluted solution. The diameter of the particles will be

$$d = \sqrt{\frac{18\,nsV}{1-Vp}}$$

where V is the specific volume of the solvent and n is the density. For exact measurements the shape of the particles, other than spherical, has to be taken into account. In a first approximation, however, as in the present study, this correction can be neglected. Moreover, it could be shown that the sedimentation rates are, disregarding some exceptions, in fairly direct ratio to the molecular weights as far as proteins are concerned (Fig. 1). If the sedimentation rates are plotted against the molecular weights, using the data available in the literature *(10)*, a distinct curve results, which in spite of its limitations, can be applied to the results obtained from the ultracentrifuge.

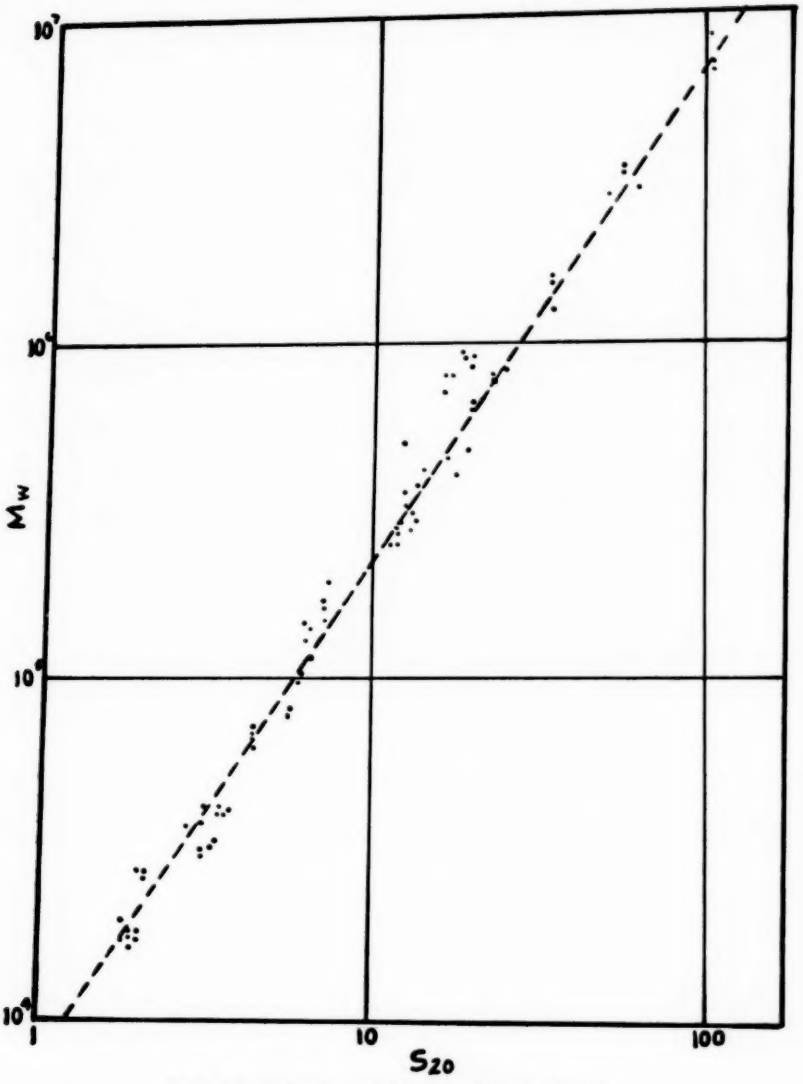


Figure 1. Molecular weight and sedimentation rate.

The honey used in these experiments was a dark buckwheat honey (Pfund grade 129) of the Finger Lakes region. It had a moisture content of 17.3%. The total amount of its colloids, determined by precipitation at the isoelectric point, filtration, and drying at 45°C. in vacuum, was found to be 0.278%.

For the runs of the samples in the ultracentrifuge the honey had to be dialyzed to remove the sugars and coloring compounds with lower molecular weight which would pass the dialyzing membrane. The procedure of dialysis was not easy since the pH

of the honey when dialyzed against distilled water adjusts itself to the isoelectric point of the colloids which are there irreversibly precipitated, thus rendering the sample useless. After many attempts the following buffer composition was found satisfactory:

11.690 g./liter NaCl 0.200 m
3.297 g./liter Na_2HPO_4 0.615 m
1.063 g./liter NaH_2PO_4 0.385 m

By dialyzing against the above buffer solution the sample became greatly diluted due to osmosis. After the dialysis in a rotating dialysator was complete, the buffer salts were removed by thorough dialysis against distilled water. The solution was then concentrated, through freeze drying, to the original quantity of honey used in the experiment. With an effective vacuum pump and by applying acetone-solid CO_2 as freezing-mixture, the frozen solution never melts during concentration. The concentrated solution was quite dispersed and the freezing did not alter or denaturize the colloidal proteins as had been feared. After it had been demonstrated that this solution would not precipitate by standing for 3 hours at room temperature, it was filled in the cell of the centrifuge. The final speed of the rotor of the centrifuge, at which the experiments were carried out, was 57,500 r.p.m. This speed was reached in 15 minutes at a load of 88 volts and a vacuum of 0.5 micron. The temperature of the rotor was 27.1°C. and a total of 12 experiments were made. The diffractive index boundary photographs were taken with 20 seconds' exposure time, using a filter of 5,700A. The rotor factors, necessary for the calculations, are listed in Fig. 2 and the formula for computing the sedimentation rates is

$$s_{20} = \frac{Fx}{xm} \cdot \frac{1}{t} \cdot \frac{Ht}{H_{20}} \cdot \frac{1K}{w^2}$$

where

s_{20} = sedimentation rate reduced to 20°C. = s × 10^{-10} = Svedberg unit
K = viscosity and density correction for buffer solution = 1.073
Ht/H_{20} = viscosity ratio of water
w = angular velocity of rotor = $1/w^2 = 2.55 \times 10^{-8}$
F = factor for the optical enlargement in the system = 0.434
xm = median difference between two $Y-F(C_1-x_{50})$ values of Tables 1, 2, 3
x = distance of migration of boundary in the cell
t = time between experiments, in seconds.

Experiment No. 9 was chosen for computing the relative amounts of the colloidal systems as they appear in three boundaries (Fig. 3), two of which are distinctly colored. The picture of this exposure was projected on a graph paper and traced. Its median line has been exactly calculated and the base line estimated (Fig. 4). Since the total

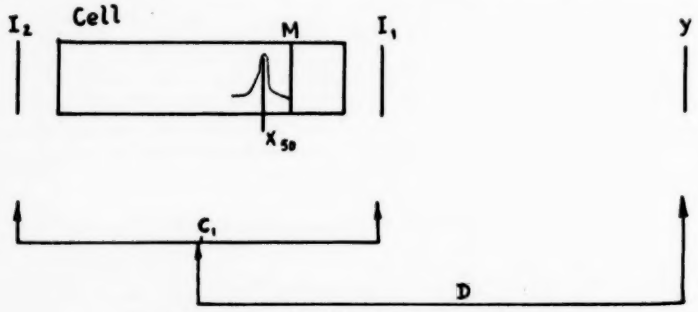


Figure 2. Factors for the ultracentrifuge.

I_1: index 1
I_2: index 2
M: meniscus
x_{50}: maximum of boundary
Y: center of rotation
C_1: distance I_2-I_1
D: C_1-Y, 6.5 cm.
$M-I_1$: 0.82 in.
C_1-I_1: 1.82 in.
C_1-M: 1.02 in.

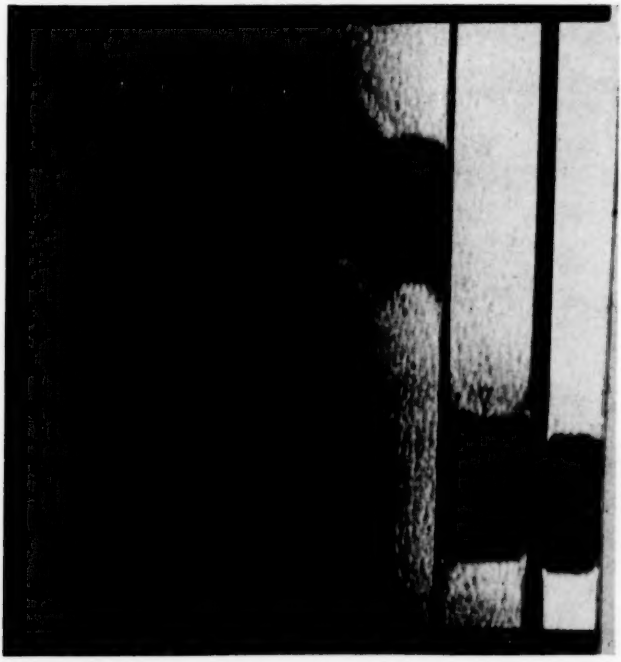
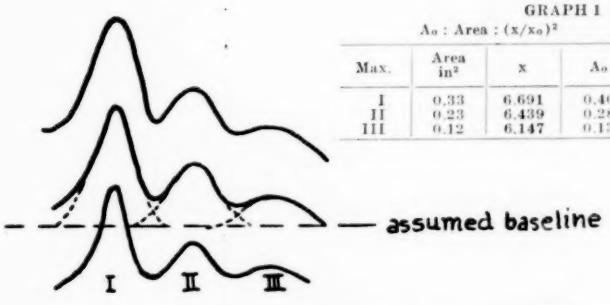


Figure 3. Refractive index (Schlieren picture) taken with a Spinco analytical ultracentrifuge.

Concentration: 0.278% in distilled water
Speed: 57.5 × 10^3 r.p.m.
Bar angle: 35°
Exposed: 32 min. after full speed
Exp. time: 20 sec.
Filter: 570 m. micra
Rotor temp.: 27.1°C.



GRAPH 1

A_o : Area : $(x/x_o)^2$ x_o : 6.057

Max.	Area in^2	x	A_o	$x_{50}-M$	%
I	0.33	6.691	0.40	1.46	49.4
II	0.23	6.439	0.28	0.88	34.6
III	0.12	6.147	0.13	0.27	16.0

Figure 4. Diffraction index (ultracentrifuge).

TABLE 1

Data for the determination of sedimentation rate for boundary maximum I.

Experiment	t sec.	Temp. °C.	Bar angle	In $x_{50}-M$	In Δx	In cell $F(c_1-x_{50})$	$6.500-F(c_1-x_{50})$	Xm	$F\Delta x$	$\frac{F\Delta x}{Xm}$	$\frac{ht.10^{-4}}{h_{20}}$	S_{20}
3	240	26.9	50	0.30	.....	0.312	6.188	.....	.....	.....	8496	.....
5	480	26.8	50	0.55	0.25	0.204	6.296	6.242	0.1085	0.0173	8516	8.35
6	480	26.8	40	0.77	0.22	0.108	6.392	6.344	0.0956	0.0150	8516	7.23
7	480	26.7	40	1.00	0.23	0.009	6.491	6.4415	0.1000	0.0155	8535	7.50
8	480	26.6	35	1.24	0.24	−0.095	6.595	6.543	0.1040	0.0159	8555	7.85
9	480	26.6	35	1.46	0.22	−0.191	6.691	6.643	0.0956	0.0144	8555	7.05
10	480	26.5	35	1.72	0.26	−0.304	6.804	6.7475	0.1128	0.0167	8574	8.10
											Average....	7.68

TABLE 2

Data for the determination of sedimentation rate for boundary maximum II.

Experiment	t sec.	Temp. °C.	Bar angle	In $x_{50}-M$	In Δx	In cell $F(c_1-x_{50})$	$6.500-F(c_1-x_{50})$	Xm	$F\Delta x$	$\frac{F\Delta x}{Xm}$	$\frac{ht.10^{-4}}{h_{20}}$	S_{20}
6	.....	26.8	40	0.48	0	0.234	6.266	.....	.....	.....	8516	.....
7	480	26.7	40	0.61	0.13	0.135	6.365	6.3155	0.0563	0.00893	8535	4.33
8	480	26.6	35	0.75	0.14	0.108	6.392	6.3785	0.0608	0.00953	8555	5.63
9	480	26.6	35	0.88	0.13	0.061	6.439	6.4155	0.0563	0.00880	8555	4.21
10	480	26.5	35	1.02	0.14	0.000	6.500	6.4695	0.0608	0.00936	8574	5.55
11	1119	26.5	35	1.40	0.38	0.165	6.665	6.5825	0.1650	0.02510	8574	5.26
											Average....	4.80

TABLE 3

Data for the determination of sedimentation rate for boundary maximum III.

Experiment	t sec.	Temp. °C.	Bar angle	In $x_{50}-M$	In Δx	In cell $F(c_1-x_{50})$	$6.500-F(c_1-x_{50})$	Xm	$F\Delta x$	$\frac{F\Delta x}{Xm}$	$\frac{ht.10^{-4}}{h_{20}}$	S_{20}
9	.....	26.6	35	0.27	0.75	0.326	6.174	.....	.....	.....	8555	.....
10	1119	26.5	30	0.36	0.11	0.286	6.214	6.194	0.0488	0.00787	8574	1.17

amount of the colloids in the honey is known, this graph (Fig. 4) will give the specific ratio of the colloids in this particular honey sample.

The sedimentation rates for the 3 boundary maxima are computed from the data accumulated in Tables 1, 2, and 3 and show the following results:

Boundary maxima	S_{20} average	Relative amounts	Absolute amounts
I	7.68	49.4	0.138
II	4.85	34.6	0.096
III	1.17	16.0	0.044
		100.0%	0.278%

These figures will be slightly higher if the concentration of the colloids is extrapolated to zero.

When these sedimentation rates are graphed (Fig. 1) the following molecular weights are found:

Boundary maxima	Mol. weight found
I	146,000
II	73,000
III	9,000

It can be shown that most protein molecules have molecular weights which are multiples of about 17,600. Consequently, boundary III is unlikely to represent a protein, but could be a polysaccharide. Using the multiple 8 for the I boundary and multiple 4 for II, fair congruence with the found data can be observed.

Multiple of 17,600	Mol. weight theoret.	Mol. weight found	Error %
8	140,800	146,000	3.8
4	70,500	73,000	3.3

The error can be called small in view of the fact that some corrections have been omitted from the calculations.

It is possible, however, that two protein molecules of different size and shape would have identical frictional coefficients and would migrate with the same sedimentation rate. Consequently, they would not show different boundary maxima. To lessen the probability for such a coincidence the dialisate of the same honey sample was analyzed also by electrocataphoresis. In this case the buckwheat honey was dialyzed with M/100 phosphate buffer of pH 7.55.

In connection with the cataphoretic studies the mobility of the honey colloids could be determined if the electrical conductivity is established. This has been found to be 9,800 ohms, with a cell constant of 12.74. The mobility is then

$$p = -\frac{x \cdot l \cdot g}{i \cdot t} T$$

where

x = distance of movement in cm. = 0.20
l = conductivity in reciprocal ohms = 0.0013
g = cross-section of cell in $cm.^2$ = 0.75
i = amps = 0.005
t = time in seconds = 600
T = temperature correction.

For the honey sample in question

$$p = -6.5 \times 10^{-6}$$

The electrocataphoresis was carried out in a Tiselius instrument with an optical system similar to that of the ultracentrifuge. To reduce thermal currents or eddies in the cell it was kept in a temperature controlled bath of 4°C. The magnification ratio of the instrument is 2.02 and 0.005 amp. current was used at 1,100 volts.

Throughout the experiments 3 distinct boundary maxima could be observed, and a photograph of them was projected with 7.5 × magnification on paper, where it was traced and planigraphed (Fig. 5).

The ratios of the 3 colloidal components as obtained by the ultracentrifuge and by electrocataphoresis are in fair congruence:

Max.	Ultra-centrifuge	Electro-cataphoresis
I	49.4	47.5
II	34.6	37.2
III	16.0	15.3

The dialyzate of the buckwheat honey sample, was precipitated by adjustment of the pH and boiling. The precipitate, analyzed for total nitrogen, gave 13.8%. If it is assumed that boundary III is not a protein, but probably a polysaccharide, it can be expected that the nitrogen content of the mixture will be 13.5%, which coincides fairly well with the found figure.

For a further check on the molecular weights of the colloidal components of dark buckwheat honey the refractive index increment and the light scattering of the dialyzate

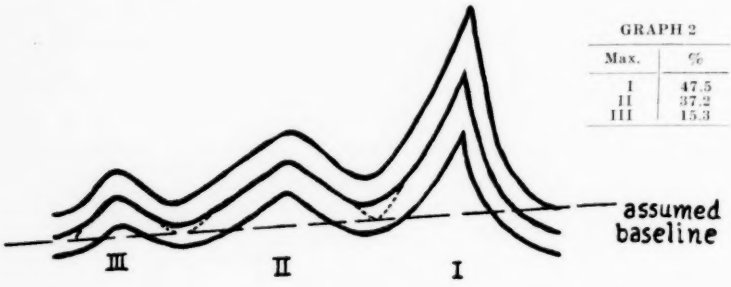


Figure 5. Diffraction index (electrocataphoresis).

has been determined. It could not be expected, however, that the result would yield a coherent answer since this colloid is a mixture of greatly different sizes. But the figure, found to be about 100,000, is in the order of magnitude of the molecular weight of the major components as has been determined and mentioned above. After the separation of the mixture into its components has been accomplished this method can be applied again to the separated constituents.

For the same reason the infra-red spectrum of this colloid mixture did not reveal much either. The spectrum of the colloid, which was precipitated in vacuum on a silver chloride plate, is fairly simple. Some of the major maxima could be identified as representing the N-H bond and the peptide-chain linkage, others are C-H stretching and bending bonds.

SUMMARY

From the above data, as shown by electrocataphoresis and the ultracentrifuge, it is indicated that the colloidal constituents of buckwheat honey consist of three main components, two of which are uniform disperse colored proteids or cenapses with molecular weights of about 73,000 and 146,000, respectively; the third appears to be a polysaccharide of the molecular weight of 9,000. Further work on the separation and identification of these colloidal constituents is in progress.

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THE AMOUNTS OF SIX B-VITAMINS IN FRESH AND DRIED FIGS

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In 1935 Morgan, Field, Kimmel, and Nichols *(15)* determined the vitamin content of figs of 4 varieties and 3 crop years by the biological assay methods then available. Quantitative values for thiamine and carotene and comparative values for riboflavin and ascorbic acid were obtained. Losses due to various methods of drying and processing were found through comparison of the values of comparable fresh and dried samples. Particular attention was given to the effects of varying amounts of sulfur dioxide treatment. For the first time it was demonstrated in these and similar studies of grapes and raisins by Morgan, Kimmel, Field, and Nichols *(16)* that sulfuring produced extensive destruction of thiamine. No such effect was noted for riboflavin, and protection rather than destruction was reported for ascorbic acid and carotene. No earlier vitamin studies of figs had been found, nor has any later notable examination of the fruit been reported.

The present study included determinations of thiamine, riboflavin, niacin, pantothenic acid, vitamin B_6, and folic acid in 4 varieties of figs of 2 or 3 crops, and in some cases a comparison of chemical and microbiological with biological methods of assay. The continuing importance of such comparisons, particularly with foods of low potency, has been pointed out previously by Jentsch and Morgan *(9)*.

SAMPLING PROCEDURE

Samples of Adriatic, Calimyrna, Kadota, and Mission figs grown in 1949, sundried and commercially processed, were first analyzed. No corresponding fresh fruit was available nor were the details of processing obtainable. Seedless Calimyrna figs commercially processed were also included in these assays.[a] These samples represent the dried figs which reach the retail market. In addition, other samples of fresh and dried figs of the crop of 1949, which were laboratory processed, were procured in the manner described below for the 1950 crop and thiamine and riboflavin analyses were made on them.

In 1950, samples of these same varieties were gathered dried from under the trees in the Fresno area and fresh fruit from the same trees was also obtained.[b] The dried fruit was heated[c] at 100°C. for 4 to 12 minutes in a water extract of 10°B. made from similar figs to obtain the desired degree of rehydration, then steam heated by bringing the pressure to 15 lbs. after which the steam was turned off. This produced fruit with moisture content of 33 to 38%. The fresh samples were wrapped in vapor-proof pliofilm packages and kept frozen. The processed figs were ground thoroughly, mixed, and representative samples wrapped in similar pliofilm and kept frozen.

Another set of samples of the same varieties was obtained in 1952 from the same trees. These were analyzed fresh, sundried, and laboratory processed for vitamin B_6.[d]

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[a] The seedless figs were provided by Mr. B. D. Innes, Planada, California.

[b] The samples were provided by Dr. Robert M. Warner, director of research, California Fig Institute, Fresno, California.

[c] The processing was done by the Division of Food Technology, College of Agriculture, University of California, Berkeley.

[d] Aileen Thielen did most of the assays for vitamin B_6.

METHODS

Moisture determinations were done by drying *in vacuo* at 52°C., nitrogen by macro-Kjeldahl, and ash by charring followed by incineration in a muffle furnace at 980°C. Six determinations in duplicate of moisture, nitrogen, and ash were usually carried out on each sample. This was true also of the vitamin assays.

Riboflavin. The microbiological method of Snell and Strong *(22)* was used as well as the fluorometric method of Peterson, Brady, and Shaw *(19)*. Low actinic glassware was used throughout this determination. Close agreement of these methods was found in the fresh samples but interfering pigments so decreased the accuracy of measurement of fluorescence in the processed figs that agreement with the microbiologically obtained values was less satisfactory.

Thiamine. The thiochrome method, a composite of several published methods including those of Hennessy and Cerecedo *(5)* and Conner and Straub *(4)* was used for measuring the thiamine. Samples were extracted with 0.1 N H_2SO_4 and autoclaved 15 minutes at 15 lb. A comparison was made of the use of the autoclave and water bath for thiamine extraction and no difference was found.

Extracts were adjusted to pH 4.5 with 4 M sodium acetate, 5 ml. 7% Clarase in acetate buffer added to the fresh fig preparations, and 15 ml. 2% Pectinol 10 M (Phaff and Joslyn, *20*) in addition to the Clarase to the processed samples. This was necessary in order to obtain a satisfactory filtrate in the latter case. Incubation was carried out at 37°C. for approximately 20 hours, the extracts made to volume and filtered. The vitamin was adsorbed on a column of activated Decalso and the fluorescence of the isobutyl solutions read on the Coleman electronic photofluorometer Model 12.

Niacin. Niacin determinations were made by the microbiological method of Snell and Wright *(23)*. Extraction was effected with 0.1 N H_2SO_4 followed by autoclaving 30 minutes at 15 lb., cooling, adjustment to pH 6.8 and to volume, and filtering. *L. arabinosus* 17-5 was used for the microbiological assay.

Pantothenic acid. The extraction procedure for pantothenic acid was that of Ives and Strong *(7)*. *L. arabinosus* 17-5 was used for the microbiological assay (Skeggs and Wright, *21*). The standard used was calcium pantothenate dextrorotatory* corrected by factor 0.92 for pantothenic acid.

Since digestion experiments with a preparation of chicken liver enzyme prepared by the method of Kaplan and Lipman *(11)* and with a mixture of alkaline phosphatase and chicken liver enzyme (Neilands and Strong, *17*) produced varying and inconsistent results and also registered high enzyme blanks, the Mylase digestion procedure was thereafter adopted.

Folic acid. The extraction procedure for folic acid was that of Bird *et al. (2)* using hog kidney enzyme. Tomato juice agar and tomato juice broth (Lyman *et al., 12*) were used for stock culture and culture medium for *Streptococcus faecalis* 8043. Bacto dehydrated medium prepared according to the formula of Capps, Hobbs, and Fox *(3)* was used.

At the beginning of this study a chicken pancreas enzyme prepared by the method adopted by Mims and Laskowski *(13)* was used to release additional microbiologically available folic acid. Approximately the same values were obtained with this as with the hog kidney. The use of hog kidney enzyme was adopted however because of the high enzyme blanks produced with the chicken pancreas preparation.

Vitamin B_6. The microbiological procedure of Atkin, Schultz, Williams, and Frey *(1)* was used with saccharomyces carlsbergensis 4228 as the organism. The standard was anhydrous crystalline pyridoxine hydrochloride.

BIO-ASSAY METHOD

All of the bio-assays were conducted similarly and according to the method of Jentsch and Morgan *(9)*. The basal diet contained in percent, vitamin-free casein 22.0, fat (Primex) 9.0, and salts, Hubbell *et al., (6)* 2.5. The vitamin supplement provided per rat per day, 20 μg. each of thiamine, riboflavin, pyridoxine, and folic acid, 100 μg. each of calcium pantothenate and p-aminobenzoic acid, 66 μg. of nicotinic acid, 2.5 mg. of inositol, 5 mg. of choline, 100 I.U. vitamin A, 10 I.U. vitamin D, and 1 mg. mixed

*Procured from Merck and Company, Rahway, New Jersey.

tocopherols. After the necessary depletion period, the vitamin in question was still omitted from the supplement for the deficient groups, and several levels of the same vitamin were fed to groups of control rats. In addition 2 or more groups were fed a carefully calculated and weighed dose of figs. The growth observed over 28 days of the test period was used as the criterion of the presence of the vitamin. A standard growth rate curve was plotted for the groups receiving known intakes of the vitamin and the amount in the figs read off from this.

Thiamine, riboflavin, and pantothenic acid assays were satisfactorily obtained by this method. Pyridoxine may be determined equally well by the method. In the case of pantothenic acid the depletion diet was fed the mother and young when the young were 14 days of age instead of to the young after weaning at 21 days of age. This provided early depletion—on the average in 16 days. The standard groups were given 10, 20, 30, or 50 μg. pantothenic acid daily. The levels used in the thiamine and riboflavin assays were 3, 6, or 10.

RESULTS

In Table 1 the proximate composition of fresh and processed dried figs of the 1950 crop is presented. When the protein values were recalculated on the dry basis, it was evident that the dried fruit had lost a small amount of nitrogen through leaching into the processing extract or had absorbed sugars from the extract, thus diluting the protein content. The Kadota figs had slightly less protein and ash than the other 3 varieties.

In Table 2 all the vitamin data are shown along with an estimate of the losses produced in drying and processing. The destructive effect of SO_2 on thiamine was again seen in 2 commercially processed 1949 samples. The amount of SO_2 retained in the fruit was not determined, but this had been shown earlier to control the amount of destruction of thiamine (Morgan *et al.*, *15*).

Thiamine. In the fresh state the 4 varieties of figs contained by thiochrome determination amounts of thiamine, 0.17 to 0.27 mg. per 100 g. solids (Table 2), the mean being 0.22. In fresh fruit containing 72%

TABLE 1

The proximate composition of fresh and processed dried figs, 1950 crop

Fig sample	Moisture	Ash	Protein (N×6.25)		Carbohydrate by difference[1]
			Moist basis	Dry basis	
	%	%	%	%	%
Adriatic					
Fresh	73.5	0.71	1.17	4.41	24.6
Dried	36.4	1.84	2.47	3.88	59.3
Calimyrna					
Fresh	67.0	0.98	1.45	4.40	30.6
Dried	33.0	1.90	2.79	4.16	62.3
Kadota					
Fresh	73.9	0.56	1.00	3.84	24.5
Dried	37.6	1.46	2.34	3.75	58.6
Mission					
Fresh	71.3	0.73	1.40	4.88	26.6
Dried	36.6	1.58	2.73	4.30	59.1

[1] The fat content of fresh figs, 0.4%, and of dried figs, 1.4%, was not usually determined.

water the value was 0.06. The dried fruit contained 0.09 to 0.15 mg. per 100 g. solids, mean 0.124. In processed fruit containing 35% water the amount was 0.08. The loss in drying and processing was on the average 42%. The thiamine content of these fresh and dried figs compares favorably with those reported for beef, chicken, eggs, milk, fish, nearly all fruits and vegetables and breads and in general is exceeded only by whole cereals, legumes, nuts, pork, lamb, prunes, unsulfured raisins, and 2 or 3 of the dark green leafy vegetables (Watt and Merrill, *24*). The bio-assay values for thiamine in figs were generally 50 to 60% higher than those discussed above.

Riboflavin. In the fresh fruit the fluorometric and microbiological assays yielded values which agreed well, 0.17 to 0.21 mg. per 100 g. solids, mean 0.19. Less agreement was found in the dried samples with the fluorometric values generally higher, due probably to pigment changes. The lower values were chosen for presentation in Table 2. These varied from 0.08 to 0.14 mg. per 100 g. solid, mean 0.11. The loss in drying averaged 40%. No significant differences among the varieties emerged. In typical fresh and processed dried fruit of the usual water content the riboflavin values were 0.07 mg. % in both cases. This value compares favorably with those of most other fresh fruits and vegetables with the exception of the leafy green vegetables. Again the bio-assay values were found to be nearly double those obtained by the microbiological method.

Niacin. Fresh Mission figs contained a significantly larger amount of niacin than did the other 3 varieties, 1.88 mg. per 100 g. solids as compared with 1.31, 1.37, and 1.43. The dried samples did not, however, reflect this difference. The latter contained 0.71 to 1.08 mg. per 100 g. solids, mean 0.93. The loss in drying varied from 28 to 48%, mean 37. Typical fresh and dried fruit samples of 72 and 35% water content contained 0.42 and 0.60 mg. %. These figures are about the same as those found for most fruits and vegetables, except the dark leafy varieties.

Pantothenic acid. Pantothenic acid determinations in foods have not been made as frequently as those for thiamine, riboflavin, and niacin. Many values reported before the difficulty of extraction of this vitamin was recognized are probably low by several fold. Apparently, pantothenic acid is found in animal tissues largely in coenzyme A (Kaplan and Lipman, *11*) and separation of free pantothenic acids is difficult to effect completely. The most effective enzymatic treatments available were used in the fig analyses. The chicken liver enzyme and intestinal phosphatase did not increase the pantothenic acid yield above the mylase and clarase treatment first tried. These enzyme preparations apparently carried measurable amounts of pantothenic acid. It is possible that some of the pantothenic acid of figs is in the free state.

The fresh Adriatic and Calimyrna figs appeared to contain more pantothenic acid than did Kadotas and Missions, 1.0 mg. per 100 g. solids in the former, 1.4 in the latter. The dried samples had 0.69 to 0.97 mg., mean 0.8, showing a loss of one-third in drying and processing. In fresh and dried fruit of typical water content this indicates the presence of 0.28 to 0.4 mg. % in the former and 0.52 in the latter. These values compare

TABLE 2

Vitamin content of fresh, dried, and commercially processed figs

Variety	Crop year	Moisture %	Riboflavin		Thiamine		Niacin		Pantothenic acid		Folic acid	
			mg.%	Solids mg.%	mg.%	Solids mg.%	mg.%	Solids mg.%	mg.%	Solids mg.%	mg.%	Solids mg.%
Adriatic												
Commercially processed	1949											
Sulfured		36.5	0.09	0.14	0.02	0.03						
Unsulfured[a]		36.6	0.09	0.14	0.07	0.11						
Laboratory processed	1949											
Fresh		71.5	0.06	0.20	0.06	0.21						
Dried		29.2	0.08	0.11	0.09	0.13						
Fresh	1950	73.5	0.06	0.21	0.06	0.23	0.35	1.31	0.26	0.96	0.014	0.053
Dried		36.4	0.08	0.13	0.09	0.14	0.60	0.94	0.44	0.69	0.034	0.053
Av. % loss in drying			41		38		28		28		None	
Calimyrna[b]												
Commercially processed	1949											
Unsulfured		35.5	0.11	0.17	0.07	0.11						
Laboratory processed	1949											
Fresh		72.4	0.05	0.19	0.07	0.24						
Dried		28.1	0.11	0.15	0.10	0.10						
Fresh	1950	67.0	0.06	0.19	0.09	0.27	0.47	1.43	0.48	1.45	0.016	0.049
Dried		33.0	0.08	0.12	0.10	0.15	0.72	1.08	0.49	.75	0.047	0.070
Av. % loss in drying			40		51		24		48		None	

Kadota												
Commercially processed	1949											
Sulfured		41.1			0.02	0.03						
Unsulfured		36.4	0.10	0.16	0.08	0.12						
Laboratory processed	1949											
Fresh		68.4	0.05	0.17	0.05	0.17						
Dried		29.2	0.07	0.10	0.09	0.13						
Fresh	1950	73.9	0.05	0.20	0.06	0.21	0.36	1.37	0.36	1.38	0.012	0.046
Dried		37.6	0.08	0.12	0.08	0.13	0.44	0.71	0.61	0.97	0.033	0.058
Av. % loss in drying		40			31		48		30		None	
Mission												
Commercially processed	1949											
Unsulfured		33.7	0.05	0.07	0.07	0.10						
Laboratory processed	1949											
Fresh		71.5	0.05	0.18	0.06	0.22						
Dried		37.3	0.08	0.08	0.09	0.09						
Fresh	1950	71.3	0.05	0.18	0.06	0.20	0.54	1.88	0.31	1.07	0.017	0.059
Dried		36.6	0.09	0.14	0.08	0.12	0.62	0.99	0.51	0.80	0.043	0.068
Av. % loss in drying		48			50		47		25		None	

[a] All samples unsulfured unless designated otherwise.
[b] All values in this table were obtained with caprified Calimyrna figs.

favorably with those usually reported for fruits and vegetables (Ives *et al.*, *8*) but are much lower than those found by Jukes *(10)* by chick growth. The one rat growth assay made for pantothenic acid on figs gave a value nearly 50% above that of the microbiological determinations.

Folic acid. In no case was any decrease in apparent folic acid detected in the dried samples. In most cases an apparent increase occurred. Both fresh and dried samples of all 4 varieties contained about 0.05 mg. folic acid per 100 g. solids, with slightly larger values in the Mission samples. The apparent increases may have been due to better extraction in the dried samples perhaps due to enzymatic release of the conjugated form of the vitamin. This content of folic acid is well above that generally found in fruits, vegetables, and meats and is exceeded only by liver and the deep green leafy vegetables such as spinach, chard, and parsley (Olson *et al.*, *18*).

Vitamin B_6. The organism used has been found to utilize all 3 forms of vitamin B_6, pyridoxine, pyridoxal, and pyridoxamine just as does the rat and presumably the human. As shown in Table 5 all five varieties of figs contained about the same total vitamin B_6, expressed as pyridoxine, 0.11 to 0.15 mg. % in the fresh fruit, 0.20 to 0.27 in the sundried, and 0.17 to 0.25 in the laboratory processed samples. The loss in both drying processes was the same, about 37%. Typical fresh figs of 72% water content had 0.13 mg. % vitamin B_6 and the dried product containing 35% water had 0.32. The Mission figs yielded slightly smaller values in this as in the pantothenic acid assay.

Seedless and caprified figs. A comparison of seedless and caprified Calimyrna figs (Table 3) indicates little difference between them as to vitamin content. However, both ash and protein content appeared to be significantly lower in the seedless variant.

Comparison of biological and other assays. As shown in Table 4 bio-assays for thiamine were made on 3 samples of figs of the 1949 crop with results indicating greater thiamine activity in all cases than those obtained on the same fruit by thiochrome procedure. Four bio-assays reported on samples of the 1932 crop were recalculated in terms of thiamine from the International standard (clay adsorbate) used in that study. These are in good agreement with the bio-assays of similar samples of the 1949 crop examined by modern techniques.

Riboflavin was determined by fluorometric, microbiological, and biological means on 2 of the 1949 samples, and as has been noted previously (Morgan *et al. 14*) agreement of the values obtained by fluorometry and bio-assay was much closer than between the latter and the results of the microbiological procedure. This may be due to better extraction of the vitamin by the digestive enzymes of the rat or to the presence of some undetected growth factor in the bio-assay and to the coincidentally equal development of fluorescing non-vitamin pigments.

The one pantothenic acid bio-assay of dried Calimyrna figs also indicated better utilization of bound vitamin by the rat digestive enzymes than by the enzymatic preparation of the fig extracts for use by the microorganism. This is a reasonable result in view of the already demonstrated difficulty of separation of bound forms of pantothenic acid.

TABLE 3

Vitamin content of seedless Calimyrna figs

Variety	Moisture %	Ash %	Protein %	Riboflavin		Thiamine		Niacin		Pantothenic acid		Folic acid	
				mg.%	Solids mg.%	mg.%	Solids mg.%	mg.%	Solids mg.%	mg.%	Solids mg.%	mg.%	Solids mg.%
Seedless 1951 crop													
Fresh	72.9	0.74	0.67	0.04	0.15	0.06	0.22	0.39	1.44	0.39	1.44	0.01	0.03
Dried	35.0			0.03	0.07	0.06	0.09	0.55	0.84	0.39	0.60	0.02	0.03
Processed dried	38.0	1.37		0.06	0.09	0.07	0.11	0.52	0.84	0.45	0.73	0.03	0.05
Seedless 1950 crop													
Processed	38.3	1.39	1.28	0.08	0.12	0.01[1]		0.55	0.90	0.41	0.66	0.03	0.05
Caprified 1950 crop													
Processed	33.0	1.90	2.79	0.08	0.13	0.10	0.15	0.72	1.08	0.49	0.75	0.05	0.07
Seedless 1949 crop													
Fresh	70.1			0.06	0.20	0.06	0.20						
Dried	26.9			0.08	0.11	0.08	0.11						
Commercially processed	36.7	1.90		0.09	0.14	0.09	0.14						
Caprified 1949 crop													
Fresh	72.4			0.05	0.19	0.07	0.24						
Dried	28.1			0.11	0.15	0.10	0.14						
Commercially processed	35.5			0.11	0.17	0.07	0.11						

[1] Dried with butane gas, possibly containing SO_2. These samples were found to contain 3.5 times as much sulfur as did the corresponding 1951 samples.

TABLE 4

Comparison of biological with chemical and microbiological assays of figs

Variety	Treatment	Crop year	Vitamin	Value by bio-assay mg.%	Value by chemical or microbiological assay		
					Method	mg.%	% of bio-assay value
Adriatic	Fresh	1932	Thiamine	0.074			
	Dried unsulf.			0.156			
	Dried sulf.			0.075			
	Dried unsulf.	1949	Thiamine	0.120	Thiochrome	0.068	57
	Dried sulf.			0.054	Thiochrome	0.023	43
	Dried unsulf.	1949	Riboflavin	0.169	Fluorometric	0.163	96
					Microbiological	0.094	56
	Dried sulf.	1949	Riboflavin	0.208	Fluorometric	0.144	69
					Microbiological	0.094	45
Kadota	Fresh	1932	Thiamine	0.078			
	Dried unsulf.	1949	Thiamine	0.133	Thiochrome	0.083	62
Calimyrna	Dried unsulf.	1949	Pantothenic acid	0.70	Microbiological	0.49	70

TABLE 5

Pyridoxine (vitamin B_6) content of fresh, sundried, and processed figs (1952 crop)

Variety	Moisture %	Vitamin B_6		Loss %
		Mg.% fresh	Mg.% solids	
Adriatic				
Fresh	70.6	0.14	0.49	
Sundried	25.1	0.23	0.31	36
Laboratory processed	32.8	0.19	0.29	41
Kadota				
Fresh	75.3	0.14	0.58	
Sundried	21.5	0.27	0.34	41
Laboratory processed	31.8	0.25	0.36	38
Calimyrna				
Caprified				
Fresh	73.9	0.15	0.56	
Sundried	23.0	0.26	0.34	39
Laboratory processed	31.7	0.25	0.37	33
Seedless				
Fresh	73.9	0.13	0.48	
Sundried	19.7	0.25	0.31	35
Laboratory processed	34.0	0.22	0.34	30
Mission				
Fresh	70.6	0.11	0.40	
Sundried	23.7	0.20	0.27	33
Laboratory processed	34.0	0.17	0.26	36

SUMMARY

Thiamine and riboflavin were determined in market samples of dried processed Adriatic, Calimyrna, Kadota, and Mission figs and in comparable samples of fresh and dried fruit of the 1949 crop. These vitamins and also niacin, pantothenic acid, and folic acid were determined in orchard samples of fresh and dried figs of the same varieties of the 1950 crop. In addition 1949, 1950, and 1951 samples of fresh and dried seedless Calimyrna figs obtained by hormone treatment were examined for some or all of these vitamins. Vitamin B_6 was also determined in the 4 varieties of figs of the 1952 crop.

No significant differences among the 4 varieties, the crop years, or the method of processing were found except for sulfuring treatment which was again shown to be destructive of thiamine.

In fresh fruit of 72% water content and processed dried fruit of 35% water content the thiamine values were respectively 0.06 and 0.08 mg. %. This represents a loss of 42% in drying and processing. The thiamine content of this fruit compares favorably with that of other fruits and vegetables.

In similar typical fresh and dried figs the riboflavin content was found to be 0.07 mg. % in both cases indicating a loss of 40% in drying and processing.

The niacin contents were 0.42 and 0.60 mg. % with drying and processing loss averaging 37%. The fresh Mission figs appeared to carry more of this vitamin than did the other varieties.

Pantothenic acid was found at the level of 0.28 to 0.40 mg. % in typical fresh fruit and 0.52 in the dried product. These are probably lower than the true values because of the difficulty of separation of bound forms of pantothenic acid for use by the microorganisms used in the assay.

Folic acid was not lost in the drying process apparently but the amounts available to the microorganisms used in the assay were even increased in the dried samples. The amounts found in typical fresh and dried figs were 0.014 and 0.032 mg. %, respectively, a large amount when compared with values reported for most fruits and vegetables.

Vitamin B_6 was lost to the extent of 37% in both sundrying and laboratory processing. Typical fresh and dried fruit contained, respectively, 0.13 and 0.32 mg. % of vitamin B_6.

No differences in the vitamin content of seedless and caprified Calimyrna figs were found, but the ash and protein values of the seedless variant were somewhat reduced.

Biological assays, rat growth being the criterion, were made on several dried fig samples for thiamine, riboflavin, or pantothenic acid. In all cases these assays yielded apparent vitamin values one-third to one-half greater than those obtained by microbiological or chemical procedures. An exception was seen in the fair agreement of the fluorometric with the biological assays for riboflavin. Good agreement of the bio-assays of 1949 figs for thiamine with those previously reported for similar 1932 figs was noted.

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SOME STUDIES ON THE DESTRUCTION OF AMINO ACIDS IN PROTEINS CAUSED BY AUTOCLAVING IN PRESENCE OF GLUCOSE

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The reaction concerning the browning of proteins or the Maillard reaction *(18)* occurring when proteins are heated in the presence of reducing sugars has received the attention of many workers *(8, 9, 10, 11, 13, 14, 15, 19, 21, 22, 23,* and *24)* in recent years. Since, in the processing of foods, heat treatment is widely used, the extent to which the nutritive value of the product is affected by the non-enzymatic browning reaction is of considerable importance in nutrition.

Friedman and Kline *(2)* while investigating the relation of the amino acid-sugar reaction to the nutritive value of protein hydrolysates, indicated the limitations of microbiological assays in such investigations. These authors concluded from their observations that an important difference existed between the animal and the micro-organism with respect to the nutritional availability of the amino acids after their reaction with glucose. Similar views have been expressed by Baldwin *et al. (1)* and Lacy and Douglas *(12)*. Mitchell and Block *(20)* found that although the nutritive value of cereal proteins was impaired by heating, the amino acid content as determined by chemical methods was not altered. Several workers have reported the loss in the nutritive value of proteins on autoclaving with glucose. On the other hand, the observations of Mader, Schroeder, and Smith *(17)* and Schroeder, Stewart, and Smith *(25)* would show that the utilization of the nitrogen absorbed from lactalbumin, heated with lactose, was as good as that from the unheated protein.

Although the loss in nutritive value of protein on autoclaving with reducing sugar has been established, the evidence on the nature and extent of destruction of the amino acids is not entirely decisive. It therefore seemed desirable to investigate this problem by the application of paper chromatographic technique. It was of importance to determine whether other proteins also behave in a similar manner. In the present paper the results of these studies are presented.

EXPERIMENTAL

Materials. Casein (Hammersten), egg albumen (B.D.H.), gelatin (Eastman Kodak), fibrin (Difco), and pure glucose were used in these investigations.

Procedure. For each gram of protein one g. of pure glucose ($C_6H_{12}O_6 \cdot H_2O$) was added and mixed well with 0.5 ml. of distilled water. The protein-glucose mixtures were then subjected to autoclaving at 15 lbs. pressure for varying periods of time. Suitable controls were also run with protein alone without admixture with glucose. In the case of the casein-glucose mixture autoclaving was done for ½-, 1-, 2-, and 8-hr. periods at 15 lbs. pressure while other proteins were autoclaved for 2 hrs.

After autoclaving, the proteins were hydrolyzed with 6 N. hydrochloric acid (25 ml. for every g. of protein) by autoclaving at 15 lbs. for 6 hrs. Simultaneously a control

experiment was also carried out with the protein-glucose mixture without subjecting to autoclaving in order to find out the amount of loss of amino acids, if any, occurring during acid hydrolysis of the mixture. No significant loss of the amino acids was found under these conditions. These results are presented in Table 1. The excess acid was removed and the material was extracted with water and after filtration was made up to 250 ml. The total nitrogen contents of these solutions were determined by the micro-kjeldahl method. The percentage loss of nitrogen in the case of casein-glucose hydrolyzed after autoclaving was found to be less than 1.5.

Circular paper chromatographic analysis. In earlier reports *(3, 4)* from this laboratory, a new technique for the separation and identification of amino acids and its application to the study of protein hydrolysates was described. The technique was shown to possess many advantages over the widely used paper chromatographic technique for routine amino acid analysis of proteins. By adopting this procedure it is possible to detect small differences in the amino acid composition of proteins.

The development of circular paper chromatography in this laboratory and the extension of this technique for the quantitative determination of amino acids have greatly extended the scope of investigations that follow the deteriorative changes in the amino acids brought about in the proteins molecule by reducing sugars.

Procedure. The protein hydrolysates were spotted on the circumference of a circle (diam. 4 cms.) drawn with a pencil from the center of Whatman No. I filter paper (diam. 24.5 cms.). The volume of solution used for spotting was 21 μl in each case. Usually the samples of the control and experimental solutions were spotted as near together as possible for easy comparison of any changes in the intensity of the color of the bands separated on the chromatogram. After drying the spots, the chromatogram was developed with n-Butanol-acetic acid-water (40:10:10), and sprayed with 0.5% ninhydrin solution in acetone and dried at 65°C. for about 15 minutes. The multiple development technique was employed and the procedure was essentially the same as described by Giri and Rao *(6, 7)*.

Typical chromatograms showing the amino acid composition of the protein-glucose mixtures after autoclaving are shown in Figures 1 and 2.

Quantitative determination of the amino acids separated on the chromatogram. The method used was essentially the same as described by Giri, Radhakrishnan and Vaidyanathan *(5)*. The chromatogram after development was sprayed with 0.5% ninhydrin in 95% acetone and dried at 65°C. for 30 minutes. The bands were cut out and extracted with 5 c.c. of 75% ethyl alcohol containing 0.25 mg. of $CuSO_4 \cdot 5H_2O$. The optical density of the solution was measured using Klett-Summerson colorimeter with green filter (540 mμ).

From these readings the relative concentrations of the different amino acids are expressed as percentages of the corresponding amino acid concentrations in the protein hydrolysates not subjected to prior autoclaving with glucose. These results are given in Tables 1 and 2.

TABLE 1

Effect of autoclaving casein with glucose for varying periods of time on the destruction of amino acids

No.	Amino acids	Percentage of amino acid found in casein after autoclaving with glucose				Percentage of amino acids found in casein-glucose hydrolysate (not autoclaved)
		Duration of autoclaving				
		½ hr.	1 hr.	2 hrs.	8 hrs.	
1	Cystine	100.0	94.0	92.0	83.0	100.0
2	Lysine, histidine	84.0	51.0	51.0	51.0	100.0
3	Arginine	41.0	0	0	0	100.0
4	Serine, glycine, aspartic acid	100.0	92.0	99.0	97.0	96.0
5	Glutamic acid, threonine	100.0	99.0	100.0	100.0	100.0
6	Alanine	92.0	94.0	100.0	100.0	98.0
7	Tyrosine	91.0	89.0	100.0	84.0	100.0
8	Methionine, valine	100.0	92.0	98.0	95.0	99.0
9	Leucine, I-leucine	100.0	96.0	99.0	100.0	99.0

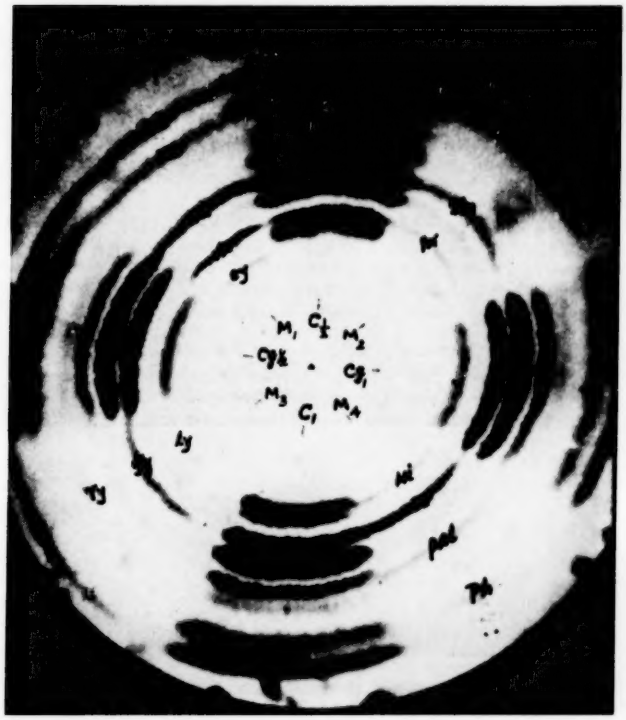

Figure 1. Circular paper mixed chromatogram showing the destruction of amino acids in casein after autoclaving with glucose.

1 g. of casein mixed with 1 g. of glucose and 0.5 cc. of water and autoclaved for varying periods at 15 lbs. and then hydrolyzed by 6N HCl by autoclaving at 15 lbs. for 6 hrs.

$C½$, C_1—Casein alone autoclaved for half and one hour respectively.
$CG½$, CG_1—Casein and glucose autoclaved for half and one hour respectively.
M_1, M_2, M_3, and M_4—Mixture of known amino acids.
M_1—cystine, arginine, threonine, methionine and iso-leucine.
M_2—arginine, glutamic acid and valine.
M_3—lysine, glycine, tyrosine and leucine.
M_4—histidine, serine, alanine and phenylalanine.

RESULTS AND DISCUSSION

It is evident from the data summarized and from Figures 1 and 2 that (a) arginine and (b) lysine or histidine or (c) both are destroyed as a result of autoclaving the casein with glucose. No significant destruction of any of the other amino acids was observed in the present experiments after one hour of autoclaving. There was a slight destruction of tyrosine and cystine after 8 hours of autoclaving in the presence of glucose as seen from the results presented in Table 1. The percentage loss observed in other amino acids are within the range of duplication limits of colorimetric determination of the intensity of the bands separated by the chromato-

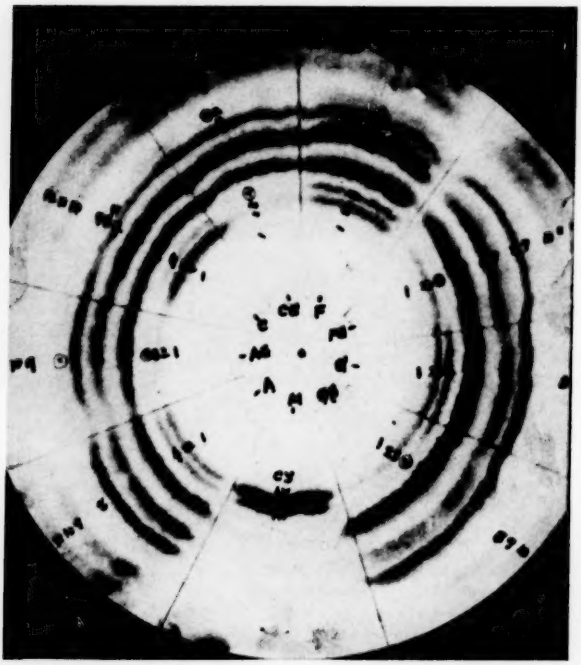

Figure 2. Circular paper mixed chromatogram showing the destruction of amino acids in proteins after autoclaving with glucose.

A, C, F, G—1 g. of egg albumin (A), casein (C), fibrin (F), and gelatin (G) autoclaved without added glucose for 2 hrs. at 15 lbs. pressure and then hydrolyzed by 6N HCl by autoclaving at 15 lbs. pressure.

AG, GG, FG, CG—1 g. of egg albumin and 1 g. of glucose autoclaved at 15 lbs. pressure and then hydrolyzed as above (AG). GG, FG, and CG refer to gelatin, fibrin and casein similarly treated.

M—Mixture of known amino acids, cystine, lysine, histidine and arginine.

TABLE 2

Effect of autoclaving of egg albumin, gelatin and fibrin with glucose on the destruction of amino acids

No.	Amino acids	Percentage of amino acids found in protein glucose autoclaved for 2 hours		
		Egg albumin	Gelatin	Fibrin
1	Cystine	100.0	93.0	100.0
2	Lysine, histidine	55.0	58.0	48.0
3	Arginine	11.0	28.0	13.0
4	Serine, glycine, aspartic acid	100.0	100.0	92.0
5	Glutamic acid, threonine	97.0	98.0	90.0
6	Alanine, tyrosine	100.0*	100.0	89.0
7	Methionine, valine	92.0	97.0	87.0
8	Leucine, I-leucine	92.0	100.0	92.0

*Alanine only in case of egg albumin-tyrosine excluded.

graphic technique. The studies with other proteins, gelatin, egg-albumin, and fibrin also show that the basic amino acids were preferentially destroyed after 2 hours of autoclaving with glucose while the other amino acids were not affected (Table 2).

Further, the results show that maximum destruction of the basic amino acids was brought about within one hour of autoclaving; longer periods of autoclaving were found to produce no further destructive action on the amino acids.

Studies with other proteins show that they differ generally from one another in the magnitude of percentage loss of the amino acids. On the basis of these results, it may be stated that only the 3 amino acids, arginine, lysine, and histidine, are adversely affected by autoclaving the proteins with glucose.

These results are of considerable significance in view of the recent observations made by Lowry and Thiessen *(16)* on the in vitro digestion studies of casein and wheat gluten autoclaved with dextrose. These authors observed the protein-sugar complex resulting from the action of glucose on the protein on autoclaving, was digested in vitro by pepsin, chymotrypsin, and pancreatin but was resistant to the action of trypsin and papain.

As amino groups in the side chain of the protein are important for the hydrolysis of the protein by trypsin, blocking or destruction of these groups will affect the action of enzyme on the protein. Since a portion of the amino groups of lysine and arginine present in the protein is free the reducing sugar combines easily with the amino group resulting in the destruction of the amino acid on autoclaving.

Further work on the enzymatic digestibility and nutritive value of the proteins subjected to autoclaving with glucose and other sugars and of starch and other products as protective agents against the destruction of the amino acids by glucose is in progress.

SUMMARY

Casein, gelatin, albumin, and fibrin were autoclaved with glucose, and the loss of amino acids of the proteins after hydrolysis was investigated by circular paper chromatographic technique.

It was found that autoclaving casein-glucose mixture for one hour resulted in complete destruction of arginine whereas in the case of albumin, gelatin, and fibrin the loss of arginine was less than that observed with casein. The destruction of lysine-histidine was of the order of about 50% on a combined basis.

Autoclaving for a period over one hour did not result in a further increase in the percentage loss of any of the amino acids.

All the other amino acids remained intact, except tyrosine and cystine which were destroyed to a very small extent after autoclaving of casein-glucose for 8 hours.

The significance of these observations has been discussed.

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STUDIES ON THE EFFECT OF GERMINATION ON THE PHOSPHORUS VALUES OF SOME COMMON INDIAN PULSES

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Krieger, Bunkfeldt, and Steenbock *(11)* and McCance and Widdowson *(12)* observed that phytic acid phosphorus is nutritionally less available than inorganic phosphorus. Bruce and Callow *(3)* are of the opinion that hydrolysis of phytic acid to inositol and phosphoric acid is a necessary step for the absorption of phosphorus and that this is accomplished by phytase present either in the food or in the gastro-intestinal tract. Phytase has been shown to be present in the intestine of rats *(17)*. An acid phytase, possibly bacterial in origin, has been shown to be present in the human feces *(8)*. Nicolaysan and Njaa *(16)* showed a distinct depression of calcium absorption in rats and pigs by diets rich in phytic acid. Courtois and Valentino *(9)* and Boutwell, Geyer, Halverson, and Hart *(2)*, however, observed that the presence or absence of phytase in food had no effect on the availability of phytin phosphorus. Boutwell *et al. (2)* further observed that vitamin D makes phytin phosphorus in wheat bran completely available to rats. Pulses are rich in phytin. It was of interest to ascertain whether the phytin content and water soluble inorganic phosphorus content change during the process of germination. The present paper presents the results of such a study carried out with 8 species of pulses.

METHODOLOGY

The terms used in this paper are those which are defined by specified analytical procedures. Water soluble inorganic phosphorus is that which is extracted as phosphate by water alone and determined colorimetrically by the method of Fiske and Subbarow *(10)*. Phytin phosphorus was determined by extracting the phytin with N/2 hydrochloric acid, precipitation thereof as the ferric salt, and determining the phosphate liberated by digesting the precipitate with sulfuric and perchloric acids. This method is that described by McCance and Widdowson *(12)*. Total phosphorus is that which is determined as phosphate after digestion of the whole dry plant material with sulfuric and perchloric acids.

EXPERIMENTAL

Germination of the seedlings. One g. of healthy pulse was weighed and kept in dry clean petri dishes; 2 cc. of redistilled water was added on the first day and 0.5 to 1 cc. water was added from the second day. The dishes were kept away from direct sunlight.

Estimation of total phosphorus. One g. of pure dry seeds was digested with 20 cc. of sulfuric acid and perchloric acid mixture (10 : 1). The digest was diluted to 100 cc.; 5 cc. aliquot was neutralized, 0.12 cc. of 10 N. sulfuric acid was added and the volume made up to 25 cc. Total phosphorus was estimated in the solution by the method of Fiske and Subbarow *(10)*.

Estimation of phytin phosphorus and water soluble inorganic phosphorus. One g. of powdered dried pulse was crushed with water and made up to 25 cc. Five cc. of this extract was used for the estimation of water soluble inorganic phosphorus. Concen-

trated hydrochloric acid was added to the remaining 20-cc. portion to bring the concentration of acid in the mixture to N/2. The mixture was placed in a glass-stoppered bottle and shaken in a mechanical shaker for 2 hours in order to extract the phytin present. Germinated pulses were crushed and the volume was made up to 25 cc. with water. Phytin was extracted in 20 cc. extract with hydrochloric acid. Phytin in the acid extract was determined by the method of McCance and Widdowson *(12)*. The results are given in Table 1.

To the 5 cc. water extract of ungerminated or germinated pulses trichloroacetic acid was added to precipitate the proteins and phosphorus in the filtrate was estimated according to the method of Fiske and Subbarow *(10)*. The value of phosphorus thus obtained was expressed as the water soluble inorganic phosphorus values of pulses (Table 2).

TABLE 1

Effect of germination on the total phosphorus and phytin phosphorus content of pulses [a]

Botanical name	Total phosphorus (mg. per 100 g.)	Phytin phosphorus content (mg. per 100 g.)			
		Ungerminated	After germination for		
			1 day	3 days	5 days
Phaseolus mungo	356±7.7[b]	80.7±1.3	74±1.6	45±2.6	32±1.7
Lens esculenta	316±9.4	87.0±2.5	88±3.4	67±0.9	41±1.7
Cicer arietinum	351±5.2	124.0±2.0	121±3.3	111±1.7	73±1.6
Cicer arietinum (another variety)	296±4.8	74.0±0.6	69±2.0	56±1.5	33±0.6
Pisum sativum	332±2.1	91.0±1.8	62±2.4	42±2.9	28±1.1
Vigna sinensis	416±6.1	62.0±3.3	59±2.8	41±1.7	31±1.6
Cajanus indicus	368±0.5	35.0±0.4	34±2.8	34±1.3	28±2.3
Phaseolus radiatus	374±6.3	88.0±3.6	81±0.8	38±2.5	36±1.8

[a] Average of 6 observations.
[b] Mean ± standard error.

TABLE 2

Effect of germination on the water soluble inorganic phosphorus content of pulses [c]

Botanical name	Water soluble phosphorus content (mg. per 100 g.)			
	Ungerminated	After germination for		
		1 day	3 days	5 days
Phaseolus mungo	109±1.6[c]	165±5.7	190±2.9	239±3.5
Lens esculenta	12±2.5	16±0.5	22±0.4	22±0.4
Cicer arietinum	46±1.7	48±1.2	63±1.1	74±0.9
Pisum sativum	.....	.....	58±5.4	65±1.2
Vigna sinensis	19±0.4	56±1.9	107±0.6	159±1.6
Cajanus indicus	34±0.6	46±1.1	74±1.3	119±3.2
Phaseolus radiatus	58±1.3	83±3.7	88±1.3	165±3.7

[c] Average of 6 observations.

From the results shown in Tables 1 and 2 it is evident that phytin phosphorus values decrease considerably during germination while the inorganic phosphorus values increase. The increase in the latter is more than can be accounted for by the decrease of phytin phosphorus. Phytase and phosphatase activities of ungerminated and germinated pulses were, therefore, studied.

Phytase activity. Barium phytate was prepared from commercial phytin according to the method of Boutwell *(1)*. Barium phytate was converted to sodium phytate before use. Three cc. of this solution gave on analysis free phosphate corresponding to 1 mg. phosphorus and phytin corresponding to 8.8 mg. phosphorus and was used as the substrate for the estimation of phytase activity. Two cc. of Sorensen's sodium hydroxide-citrate buffer and 10 cc. of the extract, equivalent to 0.5 g. of the pulse, were added to the substrate, the pH of which was adjusted to the pH of the extract (pH 6-pH 6.4) and incubated for 24 hours. The enzyme activity was stopped by the addition of

trichloroacetic acid and phosphorus was estimated as before. Phosphorus released by the substrate itself and by the extract of the pulse without the substrate under the conditions of the experiment was also studied and these values were deducted in calculating the true release of phosphorus from phytin substrate by the phytase present in the pulse extract (Table 3).

Phosphatase activity. The phosphatase activity (Table 4) of the pulse was studied with sodium glycerophosphate as the substrate. Five cc. of a 2% solution of sodium glycerophosphate, 2 cc. buffer and 10 cc. of extract equivalent to 0.5 g. of the pulse were incubated for 24 hours. The details for the blank correction and the buffer used were the same as for the determination of the phytase activity.

TABLE 3

Phytase activity expressed as mg. phosphorus liberated from phytin substrate by extract equivalent to 1 g. of pulse [d]

Local name	Botanical name	Phosphorus released by pulse germinated for 3 days
		mg.
Kalai	*Phaseolus mungo*	0.78
Musuri	*Lens esculenta*	0.34
Chhola	*Cicer arietinum*	0.57
Motor	*Pisum sativum*	0.29
Barbati	*Vigna sinensis*	0.27
Arhar	*Cajanus indicus*	0.10
Sona mung	*Phaseolus radiatus*	0.40

[d] Phytase activity was absent in the ungerminated pulse.

TABLE 4

Phosphatase activity expressed as mg. phosphorus liberated from glycerophosphate substrate by extract equivalent to 1 g. of pulse

Local name	Botanical name	Phosphorus released	
		Before germination	After germination
		mg.	*mg.*
Kalai	*Phaseolus mungo*	2.6	5.7
Musuri	*Lens esculenta*	0.8	1.2
Chhola	*Cicer arietinum*	1.1	2.6
Motor	*Pisum sativum*	1.4	3.5
Barbati	*Vigna sinensis*	1.0	7.4
Arhar	*Cajanus indicus*	1.2	3.3
Sona mung	*Phaseolus radiatus*	3.0	5.0

RESULTS AND DISCUSSION

Pulses are a good source of phosphorus as they contain on an average 0.3% phosphorus. Table 1 shows that phytin phosphorus is present in all pulses studied and constitutes about 10 to 35% of the total phosphorus content of the pulse. The phytin phosphorus values are reduced considerably during germination while the water soluble inorganic phosphorus values increase. The increase in the latter is much more than can be accounted for from the decrease in phytin phosphorus values during germination. It seems, therefore, that phosphorus present in the pulse in other combinations breaks down to water soluble phosphorus during germination. The

phytase activity as studied seems to be absent from the ungerminated pulse while the germinated pulses show phytase activity. Phosphatase activity is present in both germinated and ungerminated pulses. Germinated pulses, however, show greater phosphatase activity; they are nutritionally superior to the ungerminated pulses owing to their increased vitamin contents *(4, 5, 6, 7, 13, 14, 15)*. Diminished phytin content of germinated pulse also indicates the superiority of germinated pulse over ungerminated pulse with regard to the presence of the available phosphorus in the pulse.

SUMMARY

The phytin content and water soluble inorganic phosphorus content of 8 species of pulses have been estimated. Phytase and phosphatase activity of the pulses have been determined. It is observed that during germination phytin phosphorus values diminish and water soluble inorganic phosphorus values increase. Phytase activity which is absent in the ungerminated pulse appears in germinated pulse and phosphatase activity is increased in the germinated pulses.

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A STUDY OF THE RELATION BETWEEN WEIGHT AND JUICE CONTENT AND COMPOSITION OF PINEAPPLE FRUITS

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A number of inquiries have been received regarding the suitability of different varieties of pineapple for the manufacture of frozen juice concentrate. More specifically, information has been sought on the relation between weight and juice content and composition in pineapple fruits. This particular question was prompted by reports of investigators that better quality in oranges is usually associated with smaller fruits. Harding and Lewis *(1)* showed that after about the middle of October the smaller fruits of early and midseason oranges are consistently higher in soluble solids than the larger ones. In Valencia oranges the differences were found throughout the sampling period (January through June). Usually the smaller fruits contained a greater percentage of acid. Larger fruits (96 and 126) contained much less juice per box than did the smaller ones (250, 216, 200, 176, and 150).

The results of Harding, Winston, and Fisher *(2)* indicated that toward the end of the season, the amount of juice in oranges (ml. per g. of fruit) tends to diminish. This was more pronounced for fruits grown on rough lemon than for those on sour orange rootstock. Since oranges produced on rough lemon stock tend to be larger and coarser than those grown on sour orange stock, the results suggest that late in the season, at least, larger oranges may be expected to yield less juice than smaller fruits.

It is the purpose of this report to present the results of studies on the relationship between weight and juice content and quality of certain varieties of pineapple. More detailed information regarding the physiology of the varieties of pineapple studied has been published elsewhere *(3, 4)*.

MATERIALS AND METHODS

The pineapples employed in these investigations were produced in Florida, harvested in the "mature green" or "market ripe" stage of maturity and were shipped by railway express to the University of Pittsburgh. The study included two lots each of Abachi, Cayenne, and Red Spanish and one each of Natal and Queen. Incomplete data on an additional lot of Natal are presented for the purpose of indicating trends. Analyses were made during the seasons of 1948, 1949, and 1950. The fruits were permitted to ripen at room temperature before sampling. When full-ripe, the pineapples were peeled and cored and then macerated in a Waring blendor. The juice was then filtered through two thicknesses of cheesecloth. When the samples had stood for about 30 minutes the corners of the cheesecloth were gathered and twisted and the remaining juice was squeezed out until air bubbles rather than juice issued from the cloth. It is realized that under commercial processing much greater pressure is exerted upon fruit pulps and that correspondingly more juice would be extracted. In the present investigation it was not practicable to extract the juice except as indicated. The method was uniformly applied to all samples so that results should offer data suitable for comparisons. Furthermore, each fruit was sampled separately in all cases and this should be of value in a statistical interpretation of the results.

Total soluble solids in the juice were determined by means of a specific gravity hydrometer, the values being converted to degrees Brix by reference to conversion tables of the official methods of A.O.A.C. Determinations of total acid were made by titrating aliquots of the juice with standard sodium hydroxide and expressing the results as citric acid. The results for ascorbic acid were obtained by combining equal volumes of the juice and 8% acetic acid and titrating with 2,6-dichlorobenzenoneindophenol.

RESULTS AND DISCUSSION

Table 1 presents a summary of the results of analyses of several lots of pineapples. Values for both juice volume and constituents of the juice are included. These results represent averages of the analyses of from 17 to 36 individual fruits in each lot.

TABLE 1

Comparison of several varieties of Florida pineapples in regard to juice volume and certain constituents of the juice [a]

Variety and year	Weight, g.	Juice volume			Total soluble solids, %	Vitamin C, mg. per 100 ml.	Total acid, g. per 100 ml.
		Ml. per fruit	Ml. per g.	Gals. per ton			
Abachi (1948)	661	196	0.29	70.0	8.9	51.9	0.70
Abachi (1950)	1,131	258	0.23	54.6	9.7	52.1	1.13
Cayenne (1948)	984	286	0.29	69.6	9.1	19.3	0.99
Cayenne (1950)	1,670	429	0.26	61.5	11.0	13.0	0.80
Red Spanish (1948)	932	246	0.26	63.2	10.3	17.6	0.92
Red Spanish (1950)	787	245	0.31	74.6	13.6	17.4	0.92
Queen (1949)	708	181	0.25	61.3	12.6	35.8	0.91
Natal (1949)	767	165	0.22	57.2	15.8	10.0	0.91
Natal (1950)	641	188	0.29	68.0	14.5	.....	.....

[a] Average of from 17 to 36 individual fruits in each lot.

The average weight ranged from 641 to 1670 g. and the juice volume per fruit from 165 to 429 ml. Since there was so much variation in the size of the fruits, a better indication of the yield of juice may be obtained by expressing the results as ml. per g., or as gallons per ton of fruits. These values showed considerable variation also. They ranged from 0.22 to 0.31 ml. per g. or from 57.2 to 74.6 gallons per ton. It will be observed that variation between lots of the same variety was as great as that between varieties. In other words, it cannot be said that one variety yields more juice than another. Differences in juice volume of the same variety in different seasons may be attributable to differences in climatic factors during the growing season. Also it is not always possible to select fruits of the same physiological maturity at the time of harvest. A more mature fruit will yield more juice than a less mature one.

Relation between weight, juice volume and quality. Statistical analyses were employed to determine whether correlations exist between weight of the fruit and juice volume as well as between weight and each of several constituents of the juice (total soluble solids, vitamin C and total acid). The results are shown in Table 2.

There was a high positive correlation which was significant between the weight of the fruit and the volume of juice it contained; the larger the fruits the greater the volume of juice that can be extracted. It appears

TABLE 2

Correlation coefficients for fresh weight of pineapples and juice volume, total soluble solids, vitamin C and total acids

Variety and year	Number of fruits	Coefficient of correlation (r) between weight and			
		Juice volume	Soluble solids	Vitamin C	Total acid
Abachi (1948)	25	+.947[a]	−.389	−.151	+.120
Abachi (1950)	23	+.754[a]	+.099	−.025	−.223
Cayenne (1948)	25	+.900[a]	−.370	+.527[a]	−.079
Cayenne (1950)	17	+.654[a]	−.332	+.191	+.106
Red Spanish (1948)	30	+.447[b]	+.170	+.280	−.415[b]
Red Spanish (1950)	30	+.560[a]	−.480[a]	+.272	−.224
Queen (1949)	26	+.895[a]	+.182	+.305	−.385[b]
Natal (1949)	17	+.908[a]	−.684[a]	+.067	+.637[a]

[a] Significant to 1% level. [b] Significant to 5% level.

therefore that large pineapples should be just as satisfactory as small ones for commercial processing and that the phenomenon of larger fruits with less juice which is frequently encountered in oranges may not be expected with pineapples. Furthermore the introduction of small but high quality fruits like Natals for blending juices should not affect the total yield of juice.

As a rule there appeared to be little correlation between weight and total soluble solids or total acid. Two lots showed a significant negative correlation between weight and solids. One lot showed significant positive and two lots significant negative correlations between weight and total acid. In each instance the significantly correlated lots represent only 2 and 3 out of the 8, and where the same variety was sampled during two seasons the significant correlations were observed in only one of the seasons. It would seem therefore that other factors are involved.

A significant positive correlation between weight and vitamin C was observed in only one lot of pineapples (Cayenne, 1948). There is usually considerable individual variation in regard to vitamin C, but in general it appears to be in no way related to weight of fruit.

CONCLUSION

In conclusion, it can be stated that pineapples can be expected to yield volumes of juice approximately in proportion to their weight. The quality of the juice, i.e., percent of total soluble solids and total and ascorbic acids, is independent of weight of fruits. For the varieties of pineapple studied in these investigations varietal differences in juice quality were not great except in a few instances. These exceptions were as follows: Natal pineapples were consistently higher in soluble solids than other varieties. The Abachi variety contained consistently higher percentages of vitamin C. The Queen variety was next highest in vitamin C content.

It is hoped that this information will be of use to processors of foods, especially where blending may be necessary for increasing the quality of certain constituents of the juice.

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CORRECTION NOTICE

The authors wish to correct a typographical error in their paper "On the Development of Off-Flavor During the Storage of Frozen Raw Peas," *Food Research* **16**, 239-244 (1951). The values for beta carotene in column 5 of Table 3 should read: 2114, 2997, 937, and 2404, instead of 211.4, 299.7, 93.7, and 240.4.

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